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THE ELECTRIC RESPONSE OF NERVE TO TWO STIMULI.

By WILLIAM R. AMBERSON[1] AND A. C. DOWNING.

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INTRODUCTION.

ZOTTERMAN (1) has recently reported that the electric response of nerve to two stimuli, when measured by the maximal deflection of a sensitive moving-magnet galvanometer, fails to reach a full doubling of the value given by the single nerve impulse until the stimulus interval is made longer than 50σ. We have continued the study of this effect, and have confirmed and extended the observations. Zotterman believed that, at the galvanometer periods which he used (1 to 2 seconds), he was securing a ballistic summation of the whole electrical discharge associated with the single nerve impulse, or with two impulses in close succession. We have ascertained that an accurate ballistic summation of the electric response of nerve is not possible by this method, since the duration of the action potential wave is usually much longer than is generally supposed, and often greatly exceeds the physical period of the galvanometer. We have found that, while the maximal deflection produced by the nerve impulse gives some information concerning its intensity it is important to study the form of the deflection as well. Our observations therefore fall into two categories. In the present paper we have assembled our observations on the effect described by Zotterman, secured by a technique similar to his, in which we have read maximal deflections by eye. In the communication immediately following we will describe the results of our study of photographic records of the form of the galvanometer deflection. These two lines of evidence supplement each other, and lead us to certain conclusions concerning the form of the action potential wave in nerve which we will develop in our second paper.

[1] Guggenheim Memorial Fellow.

The Galvanometer.

The galvanometer used in these experiments is similar to that previously described by Downing(2). Both coils and moving system have been further modified. The total resistance of the coils in series is 20,000 ohms. This high instrumental resistance corresponds approximately to that of the external circuit, including the tissue. It gives a greater sensitivity than that attained in previous models. A large damping vane is fitted to the system so that it can be critically damped at short periods. In instruments of this type, when employed with relatively short period, electromagnetic damping is unimportant; the movements of the system are practically independent of the external resistance. Air damping is therefore necessary, and is secured by adjusting the distance between the vane and the damping plate until the critical value is reached.

In such experiments as those considered in the present paper, where maximal deflections alone are to be read, the readings can be secured by the use of such a system as has previously been described, and most of our earlier observations were so made. In the later work, where photographic recording of the whole movement of the system was desired, it was found necessary to increase the stability of the galvanometer, so that the zero would be reliable enough to permit an accurate reading of the curves. The usual method employed in constructing these magnet systems has been to make the magnets as nearly alike as possible, and then to mount them on the glass rod, each complex containing the same number of magnets. As their size and strength are bound to vary slightly it is practically impossible to obtain a really good astatic system in this way. A method of making them astatic has been developed, and a system having this additional improvement has been used for the photographic recording, later to be discussed.

If a system be mounted in the usual way and suspended in a uniform field, it points in the direction of the horizontal component of the magnetic force, unless it be absolutely astatic. If we avoid the neighbourhood of magnetic objects the earth's field is sufficiently uniform for our purpose. Systems suspended in it usually point in a N.-S. direction, showing that one or other of the complexes is the stronger. By means of a compass we can determine which complex this is, or, in other words, in which complex a north-seeking pole is pointing to the north. By measuring the period of the system under these conditions we can obtain some idea of the extent to which one complex is stronger than the other.

This N.-S. component has now to be abolished, and this can be done either by the addition of a tiny magnet to strengthen the weaker complex, or by inducing opposite polarity in the stronger complex, thus strengthening or weakening one complex or the other until the two are more nearly equal. Either method requires very careful manipulation. Having nearly abolished this N.-S. component we may find, on suspending the system again, that it points E.-W. This E.-W. component, always initially present, but previously masked by the stronger N.-S. component, is due to the fact that the two complexes are not quite parallel. It is abolished by the addition of a small magnet placed at right-angles to the others. When a magnet of the right strength has been obtained to balance this component the system will again point N.-S., but will have a definitely longer period. The N.-S. component may now be still further reduced by rotating the tiny magnet in one direction, and, if due care is taken, can be finally abolished, so that the system will come to rest in any position. If care be taken in the final adjustment of these systems they may be made very reliable and as stable as a moving coil galvanometer. Even at high sensitivity they are practically unaffected by uniform magnetic fields, and are best used without a shield, since when a shield is present magnetic fields approaching the system are rendered non-uniform, and the galvanometer shows magnetic disturbances. Photographic records taken with such a system are readable to 0·1 mm.

Method.

The observations here reported have been made on the sciatic nerves of large *R. esculenta* or *R. catesbiana*. The sciatic, carefully dissected from spinal column to knee joint, is killed at the distal end by immersion of a short length in hot Ringer solution, and is then mounted in a glass chamber whose construction and dimensions are shown diagrammatically in Fig. 1. In this chamber the stimulating electrodes, *A*, are platinum wire set through the side wall. The lead-off electrodes consist of glass tubes, *B* and *C*, which fit into the bottom of the chamber through ground glass joints. These tubes are filled to the tips with agar-agar, made up in Ringer solution. Small reservoirs at the end of each tube are filled with Ringer; electrical connection to the galvanometer is completed through saturated KCl calomel half cells as shown in the figure. A counter E.M.F. from a potential divider is introduced into the galvanometer circuit to balance off the injury potential. This constitutes the only complication in the circuit, which has been kept as simple as possible. The whole chamber is supported within a metal box, which can be filled

with water, at any desired temperature, so that the chamber is completely immersed. Inlet and outlet tubes allow the admission of any gaseous mixture which is desired.

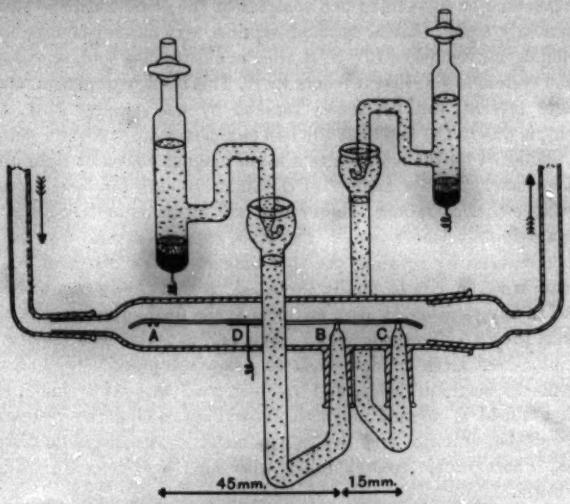

Fig. 1. Diagram of nerve chamber. See text for description.

The nerve is arranged so that the killed end is in contact with electrode *C*, and a more proximal uninjured region is in contact with electrode *B*. The distance between these two lead-off electrodes is approximately 15 mm. The nerves are so placed that electrode *B* is from 10 to 12 mm. distant from the proximal edge of the killed region. It is known that the action potential wave is diminished in size when it approaches an injured region (3, 4); this may be due to the fact that the injury current produces a condition analogous to a catelectrotonus. On the other hand it is known that, when the distance between the proximal lead-off electrode and the killed end of the nerve is sufficiently increased (2 cm. or more) the action potential wave gives a diphasic record (5). In order to avoid both difficulties as far as possible we chose to work at distances between 10 and 12 mm. The possible effect of the killed region upon our results, even at these distances, will be later discussed.

A platinum plate, *D*, 5 mm. wide and 10 mm. long, is set through the

bottom of the chamber midway between stimulating and lead-off electrodes. This plate is usually grounded, and often serves to lead to ground a physical escape from the stimulating current which may otherwise enter the galvanometer circuit. Its influence is, however, rather variable, and we are not prepared to assert that it constitutes the best remedy for such a difficulty. The chamber has been built to accommodate long nerves, so that the stimulating electrodes may be removed as far as possible (45 mm.) from the nearest lead-off electrode. This precaution has been chiefly responsible for the elimination of stimulus escapes.

The presence of such artefacts has complicated the work of many investigators. Bishop, Erlanger and Gasser(5) have considered the phenomena in some detail and have developed methods for their diminution or elimination in their apparatus. When a galvanometer is used ballistically it is not possible to distinguish directly between physiological responses and physical artefacts. By indirect methods, however, the presence or absence of such effects may be determined without great difficulty. The most convenient test is applied by the constant use of two stimuli, one of which is approximately ten times as strong as the other. The weaker stimulus, a break shock generated in one coreless induction coil, is set to be somewhat supermaximal. The stronger stimulus is supplied from a second coreless induction coil which is set to give ten times the total electrical discharge of the weaker stimulus. The relative stimulus strengths are determined by previous physical calibrations of both coils. When the single action potential waves initiated by each stimulus give identical galvanometer deflections it is a fair presumption that no escape from the stimulus is present, since such an artefact, when it once appears, is always magnified by an increase of stimulus strength. Under our experimental conditions fresh nerve almost always behaves in this manner; when this identity is not obtained the experiments are rejected.

Actual values obtained in the course of these preliminary adjustments may make this procedure more readily understood. The following readings were obtained on one nerve:

Stimulus strength (Arbitrary units)	Maximal deflection mm.
20	23, 23, 24
30	25, 25, 25
45	27, 27, 27
70	27, 27, 27
100	27, 27, 27
160	26, 27
270	28, 27, 27
685	27, 27, 26

The presence of a definite plateau at all stimulus strengths above 45 units is evident. For this experiment the two stimuli of value 70 and 685 were chosen, giving a ratio of strengths of approximately 1 : 10.

Further tests for the presence of physical distortions have been applied by the reversal of the direction of stimulation, which usually gives no change in the magnitude of the galvanometer deflection. In a group of experiments the action potentials have been suppressed by the application of chloroform vapour. When this is applied the galvanometer deflections are quickly reduced to zero, even when the two stimuli are considerably increased in strength. These tests are, we believe, more trustworthy than the usual method of crushing the tissue between stimulating and lead-off electrodes. Kato(6) has especially emphasized the fallacy involved in this supposed control.

The use of two stimuli, of which the second is ten times as strong as the first, also insures that, within the classical relative refractory period, the second stimulus will be maximal for all fibres which have recovered at all, even though their excitability be below normal. After the first 15σ maximal responses may be obtained when the second stimulus is no stronger than the first, so that the failure of action potential to recover is not associated with a diminished excitability after that time. For a considerable period we did not understand the true nature of the phenomena under investigation, and therefore continued to use stimulus strengths in the 1 : 10 ratio over the whole range of stimulus intervals, in order to be absolutely sure that our second stimulus was maximal.

The galvanometer deflection actually observed is not an absolute measure of the total amount of electricity which passes through the galvanometer circuit, even when the discharge is confined to the period for which a complete ballistic summation is possible. This apparent paradox arises from the fact that, when nerve and electrolyte solutions are in the galvanometer circuit, an initial electrical movement in one direction is succeeded by a return movement in the other direction which operates to reduce the maximal deflection. This is certainly observed when break induction shocks, sent through the agar electrodes and a length of nerve in contact with them, are then caused to pass directly into the galvanometer. The magnitude of the deflection secured then depends upon the period of time in which the galvanometer circuit remains closed. If, by the opening of a key placed in that circuit, connection is broken at various short time intervals after the generation of the shock, curves of the form shown in Fig. 2 may be secured. Thus if

the galvanometer circuit remains closed for 0·6σ a larger deflection is obtained than if it remains closed for 6σ. The reduction in amplitude of

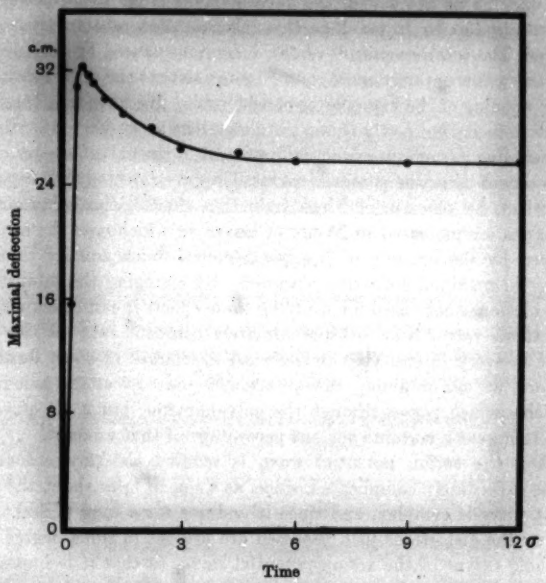

Fig. 2. Galvanometer deflections produced by break shocks of equal intensity, when the galvanometer circuit is allowed to remain closed for varying intervals of time. Ordinates: maximal deflection in cm. Abscissæ: time in σ during which the galvanometer circuit remains closed.

the galvanometer deflection to a constant plateau after 6σ is presumably caused by the development of back E.M.F.'s due to tissue and electrode polarizations, and similar effects, whose time of discharge is considerably longer than that of the original electrical movement. The tissue is not wholly responsible, since similar distortions are observed when the calomel cells are directly connected through saturated KCl, and may even be seen in all-metallic systems when large capacities or inductive windings are present.

It has not been possible directly to demonstrate that physiological E.M.F.'s are similarly affected. With injury potential always present,

and usually changing slightly from moment to moment, it is very difficult to hold the galvanometer absolutely on its physical zero, so that the mere opening of the key in the galvanometer circuit will cause small movements (up to 10 mm.) as the galvanometer returns to its zero position. The total magnitude of the deflections caused by single action potential waves is rarely more than 50 mm., so that the error introduced by the opening of the key may be considerable. We have not, therefore, been able to show directly that such distortions as we have described in the preceding paragraph are actually present in the galvanometer movements caused by action potential waves. They are detected with certainty only when, by the use of break induction shocks, the galvanometer deflections are increased to 20 cm. or larger, in which case the error introduced by the opening of the key becomes much smaller than the changes in maximal deflection observed. By changing the intensity of the induction shocks used for studying these effects it can be shown that the plateau values bear an approximately constant ratio to the peak values at every intensity. For electrical discharges of short duration, therefore, we are obtaining, not an absolute measure of the amount of electricity which passes through the galvanometer, but a set of values which represent a certain constant percentage of that amount.

When the action potential wave is studied the physical events become exceedingly complicated, since, as we shall later show, the form of that wave is complex, and since it endures for a long period. Presumably the distortions just discussed are present in some degree over the whole extent of the action potential wave, so that it is impossible to secure absolute values at any point. It is therefore evident that we cannot accept the maximal deflection of the galvanometer as an accurate quantitative measure of the intensity of the nerve impulse; it must remain merely a useful qualitative index.

We believe that the effects just described are very similar to, if not identical with, the polarization distortions observed by Bishop, Erlanger and Gasser(5). They have recognized that such distortions are probably present in the record of the action potential wave itself.

The movements of the galvanometer are made evident by focussing light from a Pointolite lamp upon a translucent scale, after reflection from the galvanometer mirror. The scale is set at a distance of 2 metres from the instrument. Readings are taken on the edge of a shadow cast by a perpendicular bar introduced into the path of the rays from the lamp. The maximum deflection can be read to the millimetre; the average of three or, occasionally, four readings is used. Scale deflections

of 25 to 80 mm. have regularly been secured from single action potential waves, when the nerve is in air, and larger movements, up to half a metre, from nerve under other conditions. Under any given set of conditions successive deflections are usually extremely consistent, and variations of no more than a millimetre in a series of determinations are the rule. As a result of this consistency the form of recovery curves can usually be determined to 5 p.c.; in the best experiments to 3 p.c.

For the shorter time intervals a Lucas spring contact breaker is used; for the longer, a Lucas rotating contact breaker. The former has been carefully calibrated from photographic records taken on a double-string Cambridge string galvanometer; the times are known to 2 p.c.

Results.

A. *The effect of temperature upon the electric response to two stimuli.*

The method of securing the data necessary for the determination of a curve of recovery of action potential can be best described in terms of an actual experiment. In Table I are given the readings secured in an

Table I. Readings to determine the recovery of action potential at 20° C.

Stimulus interval σ	Maximal deflection mm.	Stimulus interval σ	Maximal deflection mm.
0	30, 29, 30	11·44	52, 52, 52
0·71	30, 30	17·41	54, 54, 54
0·86	30, 30, 31	36	57, 57, 57
1·00	32, 32, 33	0	32, 32, 32
1·14	37, 37, 36	54	59, 60, 60
1·43	46, 45, 45	90	60, 60, 61
2·86	47, 48, 48	180	61, 61, 61
0	30, 31, 30	0	31, 32, 31, 32
5·72	50, 49, 50		

experiment done at 20° C. An inspection of these figures will show the consistency of the data. The end of the absolute refractory period is indicated by a sudden increase in the maximal deflection of the galvanometer, as the stimulus interval is gradually lengthened. In this experiment this increase appears when the stimulus interval is raised from 0·86 to 1·00σ; the end of the absolute refractory period is therefore located between these two values, and is read as 0·93σ. At 1·14σ the maximal deflection has increased by 22 p.c., and at 1·43σ by nearly 50 p.c., but from then on the rise becomes very much slower, so that at 36σ the increase is only 80 p.c. and at 90σ about 93 p.c. The stimulus interval must be made even longer than this in order to secure, as at 180σ, a full doubling of the value given by a single nerve impulse.

A correction factor, determined by physical calibration with two break induction shocks sent into the galvanometer at varying intervals, is used to correct the value of the deflections secured at intervals greater than 90σ, since beyond this point the galvanometer no longer gives an accurate ballistic summation. The use of such a correction factor for deflections given by the rather complex action potential wave is open to some objection, but it has been regularly observed that, when such factors are used, the corrected values finally rise to become double that given by the single action potential wave, within the error of the method (3–5 p.c.). Thus in the data given in Table I the average of the three readings secured at 90σ is 60·3, and this upon correction becomes 60·7; the average at 180σ is 61, and this upon correction becomes 62·8. At the end of the experiment the value for the single nerve impulse has risen to 31·5, so that the corrected value at 180σ is almost exactly double.

Changes in the value for the single impulse are almost always observed during the course of an experiment. Usually the value increases with time, but in some experiments it is found to diminish. For any

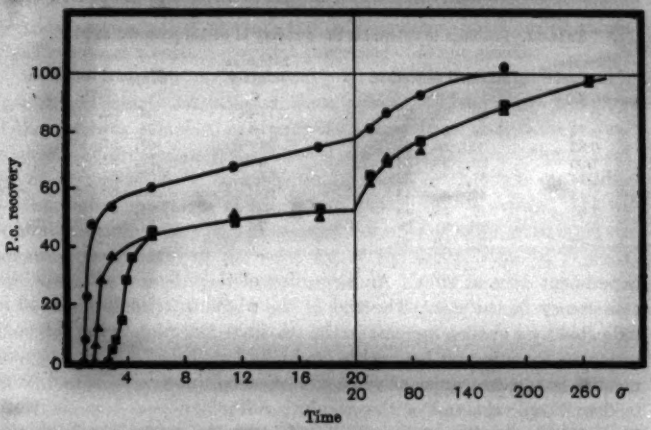

Fig. 3. Electric response at various stimulus intervals and at three temperatures. Ordinates: percentage by which the electric response to two stimuli is increased over that given by one. Abscissæ: stimulus interval in σ. Two time scales are used, so that the characteristics of the initial portions of the curves may be more clearly shown: the discontinuities in the curves are due to this.

● At 20° C. ▲ At 15° C. ■ At 10° C.

particular stimulus interval the maximal deflection of the galvanometer, given by two action potential waves, is always modified in correspondence with such changes in the base line, so that, while the absolute values may change considerably, the percentage of increase will remain nearly constant for hours, provided the physical conditions remain unchanged.

The effect of temperature upon the form of the curve of recovery of action potential is shown in Fig. 3. The curve at 20° C. is the one whose details we have just discussed. The curves at 15° and 10° C. were then secured in order from the same nerve. The magnitude of all of the deflections rises as temperature falls. Thus at 20° C. the average value for the single action potential wave was 31 mm.; at 15° C. it rose to 43 mm. and at 10° C. to 47 mm.

In general the effect of lowering the temperature is to delay the recovery of action potential. This is particularly evident in the first stages of recovery. The effect upon the duration of the absolute refractory period is profound. After the first 6σ, however, the curves at 15° and 10° C. approximate, and the later phases of recovery are practically identical. This curious result has been observed in five other experiments, carried out to confirm the observation.

B. *The effect of carbon dioxide.*

We have determined the effect of CO_2 upon the electric response to two stimuli, and find that the form of the recovery curve is considerably altered by its administration. In all concentrations above 3 p.c. it prolongs the absolute refractory period and delays recovery.

In Fig. 4 are shown the results of three determinations of the recovery curve at 18° C. The first curve (circles) represents the results obtained initially in air. The second curve (squares) represents the form of the curve found one hour later after administration of a gaseous mixture consisting of air 80 parts: tank CO_2 20 parts. The third curve (triangles) shows the results found in air about two hours later and one hour after the removal of the CO_2. The first and third curves are in good agreement.

The maximal deflection is greatly increased by the administration of CO_2. In this experiment single action potential waves gave, for the first series of readings, an average of 52 mm. For the second series, after the administration of CO_2, the average rose to 106 mm. For the third series, in air, the average fell again to 62 mm.

C. *The effect of oxygen lack.*

A few experiments have been carried out in which the recovery curve of the fresh nerve in air is compared with the curve obtained after all

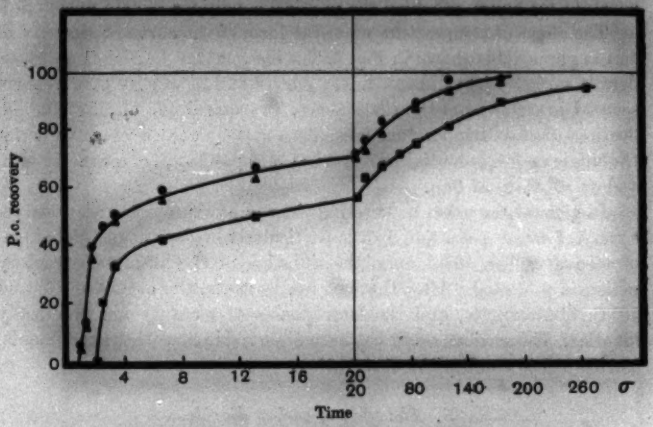

Fig. 4. Electric response at various stimulus intervals, as affected by CO_2, at 18° C. Air 80 p.c.: tank CO_2 20 p.c. Coordinate system as in Fig. 3.

● In room air at the beginning of the experiment.
■ One hour after administration of CO_2.
▲ One hour after removal of CO_2 and re-admission of room air.

oxygen has been removed. The oxygen present in tank nitrogen has been removed either by the method of Kautsky and Thiele (7) or by passing the gas over hot copper gauze in an electric furnace. This gas is then passed through the nerve chamber in such a direction that it first encounters the proximal end of the nerve. In this way any traces of oxygen which may enter through the agar electrodes are swept out of the chamber without coming in contact with the proximal portion of the nerve.

It is found that deprivation of oxygen has a relatively slight influence upon the form of the recovery curve for at least two hours after oxygen has been removed, when the stimuli are restricted to the relatively few single and double shocks necessary for the determinations. Fig. 5 presents two recovery curves determined on the same nerve (20·8–22·1° C.). The first was taken in the hour before the removal of oxygen,

the second in the second hour after its removal. A slight lengthening of the absolute refractory period is evident in the second curve, and the

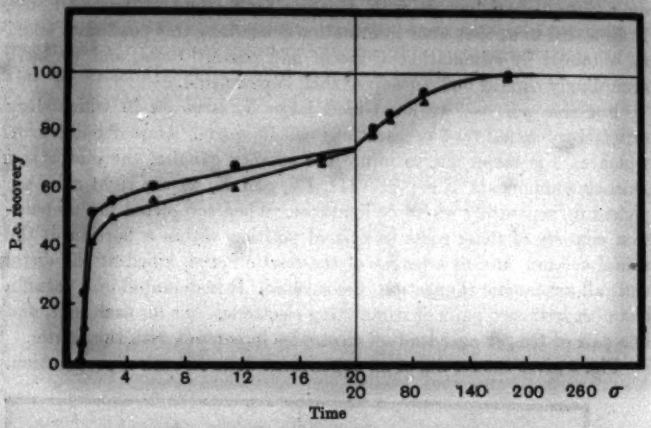

Fig. 5. Electric response at various stimulus intervals, as affected by lack of oxygen. Temperature 20·8–22·1° C. Coordinate system as in Fig. 3.

● In room air at the beginning of the experiment.

▲ During the second hour in nitrogen.

initial stages of recovery are somewhat depressed. In their later courses the two curves are identical.

The effect of oxygen lack on the duration of the absolute refractory phase will be indicated by the following values secured on this nerve which are typical of the results usually obtained:

Before N_2	$0{\cdot}8\sigma$
In N_2 for 1 hour	$0{\cdot}9\sigma$
In N_2 for 2 hours	$1{\cdot}15\sigma$
In N_2 for 2 hours 30 minutes	$1{\cdot}70$–$2{\cdot}15\sigma$

At the beginning of the third hour profound modifications in the electrical behaviour of the nerve become evident, and satisfactory recovery curves cannot be obtained after this time.

D. *The electric response of motor and sensory nerves.*

Zotterman suggests that the discrepancy observed between the time relations of the recovery of action potential as measured by his

method and the recovery of excitability as measured by the muscular summation method may arise because the latter determination involves only the quickest motor fibres, whereas his method utilizes all fibres. It appeared to us that some information concerning this possibility could be obtained by stimulation of motor and sensory roots, and we have accordingly carried out a group of such experiments.

For this purpose we have used large *R. esculenta* in which very satisfactory spinal root preparations can be made. After dissection, in which care is taken not to injure the sensory ganglia, the motor and sensory components of nerves VIII, IX, and X, on the right side, are picked up separately with two ligatures. When completed the preparation consists of these roots in normal position within a portion of the spinal column, and of a length of the sciatic nerve, killed at the distal end; all autonomic connections are severed. It is mounted in a paraffin chamber with two pairs of stimulating electrodes, one for each root, and one pair of Ringer-agar lead-off electrodes in contact with the sciatic.

Fig. 6 gives the results of an experiment done on such a preparation.

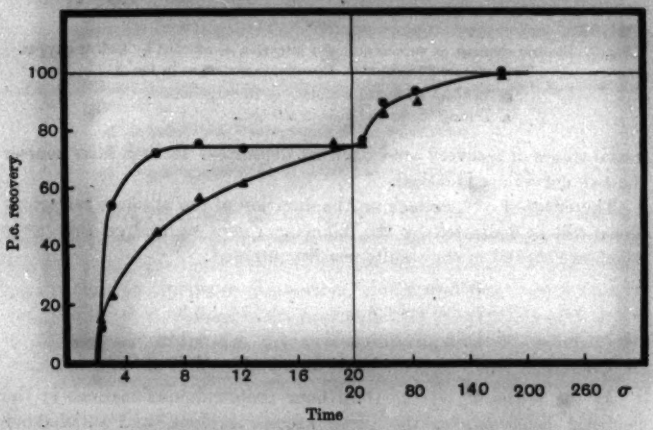

Fig. 6. Electric response in motor and sensory fibres at various stimulus intervals. Temperature 14·7° C. Coordinate system as in Fig. 3.

● Motor root. ▲ Sensory root.

It is seen that the motor root recovers very quickly, up to a plateau value, at which level the readings remain constant for a considerable

time (20–25σ) as the stimulus interval is further increased. The sensory root at first recovers more slowly, but usually gives some indication that recovery begins slightly sooner than in motor root. The later stages of recovery are practically identical. In both roots complete recovery is accomplished only when the stimulus interval is made greater than 100σ. This experiment is fairly typical of the results obtained.

The plateau on the curve of recovery of motor root is often very clearly evident. Thus in this experiment the following readings were obtained:

Stimulus interval σ	Maximal deflection mm.
0	30, 31, 31
1·5	30, 30, 30
2·3	33, 33, 34
3·0	46, 45, 46, 45
6·1	51, 51, 51, 50
9·1	52, 51, 52, 52
12·2	51, 51, 51, 52
18·6	51, 52, 51, 52
26·5	52, 53, 52, 52
29·4	52, 52, 52
33	54, 55, 55
51	56, 56, 56
0	29, 30, 30

In this series there is no significant change in the maximal deflection as the stimulus interval is increased from 9·1 to 29·4σ, although the value is definitely less than full recovery.

In these experiments it has been very evident that the maximal deflection given by stimulation of both roots at the same instant is the sum of the values given by the two roots when stimulated separately. Typical readings are as follows:

Stimulus interval σ		Single stimulus mm.	Double stimulus mm.
1·7	Motor	26	37·5
	Sensory	24	27
	Both	50	63
3·3	Motor	28	43
	Sensory	24	36
	Both	53·5	80
6·6	Motor	28·5	45·5
	Sensory	26	41
	Both	53·5	85·5

Discussion.

A summation of current, or of E.M.F., in a manner similar to that just described has been observed and discussed by various workers. Erlanger and Gasser(8), Watts(9) and Davis and Brunswick(4)

believe that this summation arises because inactive fibres act as shunts and lead away a portion of the current generated by the active fibres; an active fibre ceases to be a shunt because its own E.M.F. compensates that developed in its neighbours, and prevents current flow into it. We are not entirely satisfied that this view can explain such complete summation as we have observed, but the fact of summation is fully confirmed.

Our experiments with root stimulation show that the long delay in recovery of electric response is found in both motor and sensory fibres, and is probably a general characteristic of all nerves, although individual peculiarities are present. We were at first inclined to believe that the plateau in the curve from motor root gives evidence of the existence of a group of fibres which recover very rapidly, and to identify this group, provisionally, as efferent nerves to muscles. But with all autonomic connections cut it is difficult to see what other group of efferents can be responsible for the long delay in complete recovery. We are at present inclined to the opinion that recovery of action potential is delayed in all fibres, whether motor or sensory.

The method here used is particularly good for the determination of the duration of the absolute refractory period, and for a study of its modification with changes in temperature, and in O_2 and CO_2 tension. The absolute refractory periods shown in Fig. 3 indicate the profound effect of temperature upon the duration, and are consistent with the older observations of Gotch and Burch(10) and of Gotch(11). The relatively slight change observed in the duration of this period for several hours after the removal of O_2 is in agreement with the observations of Cooper(12) rather than with those of Fröhlich(13), who reported a marked increase, but whose results are complicated by the fact that he employed a tetanus, which Field and Brücke(14) have found greatly to increase the duration. The effect of CO_2 in lengthening the absolute refractory phase is in the same direction as that observed by Cooper(15) with the muscular summation method.

In studies of the sort here reported it is clear that we secure additional evidence that the effects of the single nerve impulse are much more persistent than has usually been supposed. Evidence pointing in this direction has been accumulating for some time and has recently been further strengthened by the discovery of a delayed heat production in nerve(16), and by the observation of Gerard Hill and Zotterman(17) that nerve, when stimulated at various frequencies, gives less heat and action current per impulse as the frequency is increased. Even when

the stimuli are as much as 50σ apart the energy liberated per impulse is definitely less than that calculated for a single isolated impulse.

In our own experiments it is not clear that the persistent modifications of the nerve are electrical in nature, although they affect the electrical response. If no other evidence were at hand we might easily infer that, long after the complete disappearance of all electrical signs of the previous activation, the living membranes are still unable to respond with a fully normal electrical discharge. This has been the usual conception of the subnormal responses long known to occur within the classical relative refractory period. Gotch(11) and Gasser and Erlanger(18) submit records, or analyses thereof, which appear to show that the second action potential wave may be both retarded in speed and diminished in size at a time when all trace of the first wave has vanished.

But the period within which such effects have previously been observed is so much shorter than the time intervals with which we have been dealing that it is difficult to believe that we are here considering the same phenomenon. Gotch could observe no delay in the second impulse when the stimulus interval was more than 5σ, at 3°–8° C., while at higher temperatures the period was even shorter. In the experiments of Gasser and Erlanger the delay has largely disappeared when the stimulus interval is as small as 2σ. The recovery of action potential is more delayed. Gotch observed a considerable diminution in the size of the second response with stimulus intervals of 4 to 7σ, but recovery seemed to be nearly complete at 8σ and can hardly have been delayed for more than 10σ at 8°–12° C. These values accord well with those obtained by Adrian and Lucas(19) with the muscular summation method, in which the relative refractory period ends at about 12σ at 15° C.

Delays in recovery of 150 to 250σ, such as are here described, are of a different order of magnitude. We finally came to believe that we must be dealing with phenomena which fall into a very different category. In a search for some explanation of these effects we were led to a study of the *form* of the galvanometer deflection given by the nerve impulse. In this study we believe that we have secured evidence which goes far to explain the long delay in the recovery curves, and which leads us to a consideration of elements of the action potential wave which are at present almost unrecognized. The results of this study will be described in the following paper.

SUMMARY.

We have confirmed the observation of Zotterman that the electric response of nerve to two stimuli, as measured by the maximal deflection of a sensitive moving magnet galvanometer, does not reach a full doubling of the value given by the single nerve impulse until the stimulus interval is made longer than 50σ. We usually obtain full recovery only after 100σ at 20° C.; at lower temperatures the recovery is even more delayed.

The effect of CO_2 is to prolong the absolute refractory period and to increase the time necessary for full recovery.

For the first two hours after the complete removal of oxygen the form of the recovery curve undergoes relatively slight modifications, although the duration of the absolute refractory phase slowly increases, and the early stages of recovery are somewhat depressed.

The long delay in recovery of electric response is found in both motor and sensory fibres. The phenomenon therefore appears to be a general characteristic of all nerve fibres.

The great difference between such recovery curves and those previously obtained by other methods is discussed and their general significance indicated.

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612.813

ON THE FORM OF THE ACTION POTENTIAL WAVE IN NERVE.

By WILLIAM R. AMBERSON[1] and A. C. DOWNING.

(From the Department of Physiology and Biochemistry, University College, London, and the Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia.)

Introduction.

The results obtained in our study of the electric response to two stimuli,

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Errata

Ordinate scales in Figures 3, 5 and 6 should read "Deflection" instead of "Maximal Deflection." In Figures 2 and 4 the readings are given as "% of Maximal Deflection," and not in "mm."

seconds. This modification in form is accompanied by [illegible] maximal deflection which may become as much as five times as great as that previously given by a single nerve impulse in air.

A similar observation was long ago reported by Waller(1) who found that the effect of expired air is considerably to augment the size of the action currents of a tetanus. Boruttau(2) and Boruttau and Fröhlich(3), using the capillary electrometer, showed that this augmentation consists of a prolongation of the action potential wave, rather than in any increase in peak voltage. Garten(4) also secured similar results. A careful study of such effects with the string galvanometer has recently been published by Davis, Pascual, and Rice(5). They state that "the

[1] Guggenheim Memorial Fellow.

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We have confirmed the observation of Zotterman that the electric response of nerve to two stimuli, as measured by the maximal deflection of a sensitive moving magnet galvanometer, does not reach a full doubling of the value given by the single nerve impulse until the stimulus interval is made longer than 50σ. We usually obtain full recovery only after 100σ at 20° C.; at lower temperatures the recovery is even more delayed.

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By WILLIAM R. AMBERSON[1] and A. C. DOWNING.

(*From the Department of Physiology and Biochemistry, University College, London, and the Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia.*)

Introduction.

The results obtained in our study of the electric response to two stimuli, considered in the preceding paper, finally led us to attempt an entirely different approach to the problem. The possibility of such an approach was first suggested by the observation that, when the nerve is surrounded by a gaseous mixture containing small amounts of carbon dioxide (*e.g.* expired air), the form of the galvanometer deflection given by a single nerve impulse is very different from that given by a break induction shock or by a nerve impulse when the tissue is in air. Even a casual inspection by eye is enough to detect the main features of this difference. It is easily seen that the time required for the galvanometer to reach maximal deflection is very much longer in the case of the nerve impulse; it is usually more than double that given by an induction shock. The return to the zero is also considerably delayed; a persistence of action potential, or at least of an E.M.F. which resembles it, can be detected for seconds. This modification in form is accompanied by an increase in maximal deflection which may become as much as five times as great as that previously given by a single nerve impulse in air.

A similar observation was long ago reported by Waller(1) who found that the effect of expired air is considerably to augment the size of the action currents of a tetanus. Boruttau(2) and Boruttau and Fröhlich(3), using the capillary electrometer, showed that this augmentation consists of a prolongation of the action potential wave, rather than in any increase in peak voltage. Garten(4) also secured similar results. A careful study of such effects with the string galvanometer has recently been published by Davis, Pascual, and Rice(5). They state that "the

[1] Guggenheim Memorial Fellow.

string...does not return to its original base line for 50 to 150σ, although normally the return is complete within 25σ or less." Our own observations differ from these previous reports only in regard to the duration of the persistent E.M.F.'s, when CO_2 is present. Using an instrumental sensitivity much higher than that employed by any of the previous workers we can always obtain "retentions" enduring for 5 seconds, and, in the extreme case have seen the galvanometer return to its zero only after 30 seconds.

The development of such persistent E.M.F.'s was so striking that we decided to photograph the whole movement of the galvanometer rather than remain content with a reading of maximal deflection, as in our earlier experiments. In the course of this photographic study we have discovered new ways of demonstrating a group of phenomena, analogous to the CO_2 retentions, which in part have previously been seen by other observers, but which were so poorly developed, and so apparently insignificant in magnitude that they have received little attention, and are, indeed, practically unknown to many present-day workers in this field. A consideration of the older literature will be given in the discussion, after we have described our own observations.

METHOD.

The method used in these experiments has been in large part identical with that described in the preceding communication, but a comparison of this method with other methods now in use for the study of nerve action potential waves has not been given, and is now in order. At first glance it may seem to students of this subject that the perfection of technique achieved in string galvanometer, capillary electrometer, and the various oscillographs, is entirely adequate to furnish us with complete information concerning the form and time relations of action potentials, and that little hope of further advance can be found in other types of electrical instruments, in which the moving parts have longer periods. This belief is undoubtedly correct if we are considering the most obvious electrical event, *i.e.* the rapid development and subsidence of a considerable difference of potential sharply localized within a few sigmata of time. With galvanometer periods ranging from 1 to 2 seconds we are obviously unable to follow the course of such rapid changes in potential. But when, in addition to this initial electrical discharge we have to do with more persistent low-voltage components of the action potential wave, we may be able to secure, through high instrumental sensitivity, a more correct and complete picture of these phases of the

wave than can be obtained by more rapidly moving but less sensitive instruments.

Even with amplification the present string galvanometer and oscillographic methods are less sensitive than the instrument which we are using. In the recent study of Davis, Pascual, and Rice(5) sensitivities up to approximately 300 metres/amp. have been used. This figure refers to the actual displacement of the string. Assuming that the projected shadow gives an amplification of 600 they reach a sensitivity of 180,000 metres/amp. By electron tube amplification, similar to that introduced by Forbes and Thatcher(6), this sensitivity can be further increased by a factor of 50, reaching 9,000,000 metres/amp. In our work the current sensitivity has always been as high as $1{\cdot}4 \times 10^{-11}$ amperes per mm. and in many cases considerably higher. This amounts to 70,000,000 metres/amp. The sensitivity of our instrument, at its worst, is therefore approximately 10 times as great. Determinations of the deflection of our galvanometer, when known constant voltages are applied through the whole system, including the nerve, show that we secure a movement of about 35 cm. per millivolt. This may be compared with the sensitivity secured, after amplification, in the cathode ray oscillograph where one millivolt gives from 3 to 10 mm. only[1].

Sensitivities of the order found in our instrument are, of course, available in the very best moving coil galvanometers, but are always associated with instrumental periods (20–40 seconds) much longer than ours. The time relations of the effects here to be described are such that an instrument with a period as long as six seconds is practically useless for their demonstration. The detection of these effects is only possible through a combination of high sensitivity with relatively brief period (1 to 2 seconds).

It will therefore be evident that our method is supplementary to the other techniques now being employed in nerve action potential work. While failing to give an analysis of the time relations of the initial electrical discharge at high voltage, in whose study these other techniques are furnishing us with a wealth of detailed information, our method is qualified to give information concerning persistent retentions at low voltage which these other methods are unable at present to detect.

In addition to the procedures detailed in the preceding paper the

[1] After writing this paper we have been informed by Dr Bishop that the cathode ray oscillograph is now being operated at much higher sensitivity. When the new technique is perfected the persistent effects here discussed should be evident in the oscillograph records.

following additions may be noted. Photographic records are taken with a Cambridge plate camera. A record of time intervals is secured in the usual manner by interrupting the light from the Pointolite lamp by a metronome. At the conclusion of the study of the action potential waves the nerves are exposed to chloroform vapour, through whose action all trace of electric response soon vanishes. The injury potential is also profoundly affected, and diminishes rapidly. After some 20 minutes, however, a new equilibrium is reached, in which the galvanometer again becomes steady. It is now possible to secure the necessary control curves. These are obtained by sending break induction shocks through the whole system, including the agar electrodes, and the length of nerve from which, during activity, the electric responses have been recorded. We thus obtain a record of the galvanometer movement produced by an electrical current of very brief duration, so short, indeed, that it is, for all practical purposes, instantaneous. We can now compare the form of the curve given by such a shock with that given by the action potential wave under various conditions. Any departure of the latter curve from the former will give qualitative information concerning the presence of potentials more enduring than those which occur in break induction shocks.

In this work the galvanometer is not quite critically damped, but is allowed to overshoot slightly. We have usually adjusted the damping so that after a deflection of 50 mm. the galvanometer overshoots by 0·5 to 1 mm. It may be well to mention again that in this instrument air damping is almost entirely responsible for the regulation of the movement. Even extensive changes of the resistance in circuit with the instrument will leave the form of the movement practically unaffected. Thus the nerve may be entirely removed and the control shock sent through the calomel cells dipping into saturated KCl. Although the maximum deflection will increase when the resistance in series is thus diminished, the curve of the resulting galvanometer movement cannot be distinguished from the control curve given with the nerve in circuit, when the maximum ordinate of the former is reduced to equal that of the latter, and all other ordinates of the first curve are reduced in the same ratio. Any change of tissue resistance produced by the administration of chloroform vapour, therefore, cannot affect the validity of the control curves later obtained. This is a virtue not possessed by moving coil galvanometers, where electro-magnetic damping is of much greater importance.

Results.

A. *The "after-positivity."*

Examination of our plates has shown that, when the nerve is in air, and at a temperature above 15° C., an "after-positivity" is always present. The interval between the beginning of the action potential wave and the appearance of this positive phase appears to range from 0·5 to 1 second. It may then persist for as much as 5 seconds, finally to be succeeded by a faintly developed "after-after-negativity," only observed in the very best records. While the speed and sensitivity of our instrument are sufficient to give very good demonstrations of these phases of the single action potential wave the voltages involved are very low. In the case of the after-positivity we estimate that the voltage does not rise above 40 microvolts, while the terminal negative phase is much less than this.

In Fig. 1 there is shown a particularly clear record of these persistent

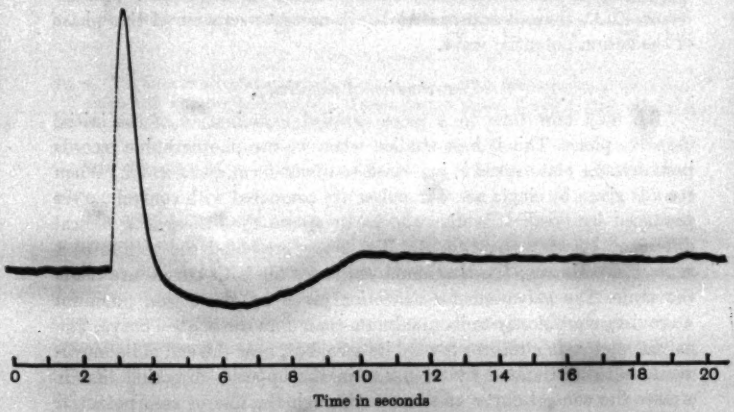

Fig. 1. Reproduction of record of galvanometer deflection given by a single action potential wave, and taken on a slowly moving plate. From the classical monophasic leads we here obtain (1) an initial electrical discharge indicating negativity of the active region, (2) an "after-positivity" enduring for about 5 seconds, (3) a faint terminal negativity. Temperature 18° C. × ½.

phases of the wave. This record was secured in London from the sciatic nerve of a large and active specimen of *R. catesbiana*, imported from

America. In order to make more evident the later phases of the curve the plate was moved slowly, at a speed of about 7 mm. per second. The characteristic features of the initial negative phase cannot be detected in such a slow record, but the later phases are well shown. In this case we are obtaining, from the classical monophasic leads, a complex curve which has three distinct phases, and which indicates a persistence of electric potential for at least 10 seconds.

It may be well to point out that the after-positivity cannot possibly be an artefact due to the use of an underdamped instrument. The control curve for this experiment shows an overshoot of 2 p.c. only (see Fig. 5 below). For a deflection of the size shown in Fig. 1 this means 1 mm. only. The galvanometer period is slightly more than 1 second; at the end of this time the control curve has returned to zero. In Fig. 1 the after-positivity reaches a maximum development of about 8 mm. deflection after 2 seconds and continues for about 5 seconds in all.

The after-positivities are well developed at 18° to 20° C. At 15° C. they are still present but very much reduced in magnitude. Not far below 15° C. they disappear. At 10° C. no trace remains of this phase of the action potential wave.

B. *The retention of negativity.*

We may now turn to a more detailed examination of the initial negative phase. This is best studied when we use photographic records in which the plate speed is increased to about 5 cm. per second. When records given by single nerve impulses are compared with control curves produced by break induction shocks it is seen that there is a radical difference between the two. In Fig. 2 are presented the results of a typical experiment; the maximum ordinates for both curves are made the same. The galvanometer deflection given by the action potential wave rises more slowly to its maximum than does the control curve. The galvanometer then returns toward its zero, but, near the end of its movement, is so accelerated by an E.M.F. in the opposite direction that it crosses the control curve and passes through the line of zero potential considerably earlier than does the control, thereafter indicating the after-positivity already described.

The delay in the attainment of maximal deflection, shown by the curve given by the action potential wave, is unmistakable evidence that a distributed potential is present, in which an element of the wave persists for at least as long as the time interval between the maximum of this curve and that of the control. In nerve removed at once from the

body and examined within the next hour this displacement may range from 60 to 150σ. It is usually about 100σ. This difference is quite

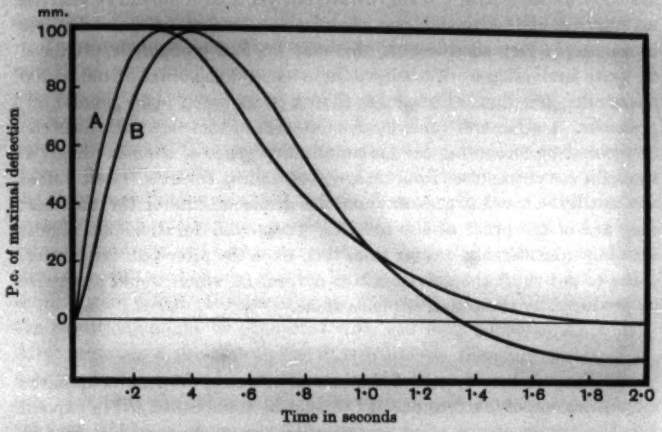

Fig. 2. The form of galvanometer deflection given by a single action potential wave compared with a control produced by a break induction shock. Curve *A*: Control. Curve *B*: Action potential wave. Temperature 17·5° C.

outside the error of reading from the plates, which is not greater than 15σ.

The conditions for the demonstration of this effect may now be stated in more detail. The prime essential is that the period of the galvanometer shall be sufficiently reduced, even though this is associated with a diminished sensitivity. We have found that, when the control curves rise to their maximum deflection in 0·2 to 0·3 second, the single action potential wave regularly gives shifts of the magnitude above stated, so that in one record clear evidence for a persistent negativity, succeeded by an after-positivity, can be secured. When, with a more sensitive instrument and longer period, the control time to maximum is 0·5 second or longer, the after-positivity may develop before the maximum is attained. In such a case the action potential wave may give a maximal deflection actually sooner than the control, the initial phase of the wave being partially masked by the second phase. Even in such an experiment, however, it is evident that the galvanometer rises more slowly at first, when the nerve impulse is being studied.

Concerning the voltages and time relations of these negative retentions we are as yet able to make no satisfactory quantitative statement, since, as we shall later discuss, we are not yet able to do curve analysis on our records. Although the modification in form of galvanometer movement is very considerable, this may be, and probably is, produced by quite low voltages. We believe these to be of the order of 100 to 200 microvolts, but this is little more than a guess based upon general impressions. A minimum value for the duration of this negative phase can be secured by measuring the maximum divergence of the records given by single nerve impulses from their corresponding controls. These values are usually somewhat greater than the displacements of the maxima; they are of the order of 100 to 175σ. The actual durations are almost certainly considerably greater than this, since the after-positivity intervenes to cut short the galvanometer movement which would otherwise be produced by the negative phase alone.

C. *The effect of carbon dioxide.*

We have already described the general nature of our results upon the administration of low tensions of CO_2 such as those contained in expired air. The nature of this effect as shown by our method is illustrated in Figs. 3, 4 and 6, in connection with sections D and E. The after-positivity present in the action potential wave when the nerve is in air has completely disappeared. In its place is a very prominent retention of negativity, which endures for seconds.

D. *The influence of rest and activity.*

When we attempted to extend our studies to action potential waves generated in motor and sensory roots, using a technique described in the preceding paper, we were at once impressed with the relatively slight difference obtained between the curves given by single nerve impulses and those produced by break induction shocks. Indeed in some experiments the two curves became almost indistinguishable, and in every case the persistent E.M.F.'s were manifestly very much reduced. Our first impression was that the retentions which we had been studying might possibly be developed mainly in autonomic fibres, which may be excited when the whole nerve is stimulated but which are not affected by root stimulation, since all autonomic connections have been cut. We therefore introduced a third pair of stimulating electrodes into our nerve chamber so that the whole nerve could be stimulated, as well as each root separately or both together. We now secured records which showed

definitely that when the retentions had diminished or disappeared on root stimulation a similar effect was evident in the whole nerve as well. These results indicated that some other factor than the elimination of autonomic fibres was responsible for the diminution of the retentions.

There remained one other difference between the root experiments and those previously performed—namely a difference in time of preparation of the tissue. In our earlier experiments we usually prepared the nerve and secured our records in air within the first hour after the animal was pithed. The root preparations, on the other hand, were much more difficult to make, and a delay of several hours intervened before the tissue was finally mounted and the first records taken. During this time the tissue received no stimulation other than that produced by the tying of ligatures and occasional contact with metal instruments. Even this type of excitation was practically absent in the last hour before the records were taken. It seemed to us, therefore, that the diminution of the retentions might be connected with this long period of inactivity. After securing a preliminary set of records we therefore arranged to re-activate the tissue by brief periods of tetanic stimulation. Further sets of records were then taken, after various periods of stimulation, of the form of the galvanometer deflection given by single action potential waves generated several minutes after the cessation of the multiple stimulation. The retentions now reappeared, and became more and more powerfully developed the longer the previous stimulation; the maximum deflection and the time to maximum became progressively greater and greater.

The essential features of this recovery are shown in Figs. 3 and 4. In Fig. 3 are given the forms of the curves as recorded from motor root, all set to the same time scale, and with all ordinates double those actually observed. Curve *A* is that given after long inactivity; it is almost identical with the control. *B* is the curve secured from a single action potential wave 2 minutes after a multiple stimulation lasting for 15 seconds. *C* is the curve given still later and 2 minutes after the end of a multiple stimulation lasting for 1 minute.

For an hour after this last stimulation the tissue was allowed to remain almost inactive, with occasional single stimulations only. The maximum deflection gradually decreased again, but an effect of the previous excitation was still evident at the end of the hour. The nerve was then surrounded by expired air; 10 minutes later curve *D* was secured.

In Fig. 4 these four curves are again shown, with maximum ordinates

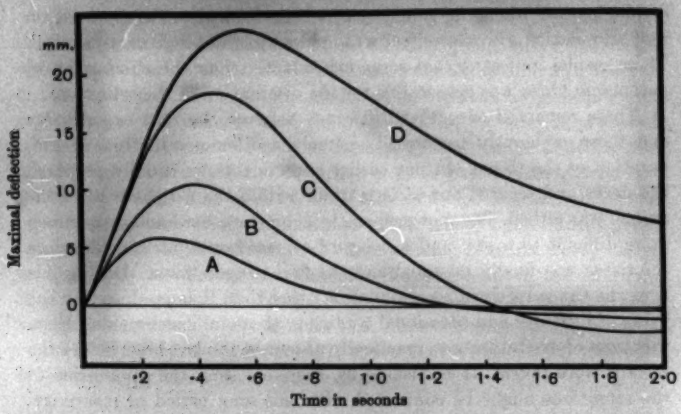

Fig. 3. Form and relative magnitude of galvanometer deflections given by single action potential waves in motor root. Curve *A*: After long inactivity. This curve is practically identical with the physical control curve. Curve *B*: Two minutes after cessation of tetanic stimulation lasting for 15 seconds. Curve *C*: Two minutes after cessation of tetanic stimulation lasting for 1 minute. Curve *D*: One hour later and 10 minutes after the application of expired air. Temperature 17° C. ×2 (for ordinates).

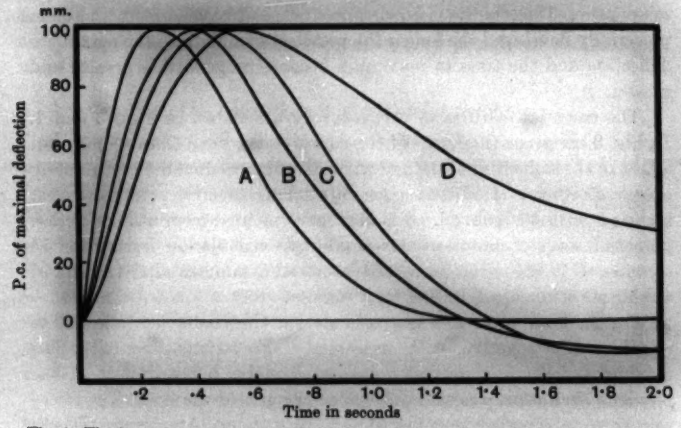

Fig. 4. The four curves of Fig. 3 are here set to the same maximum ordinate, in order to show more clearly the displacement of the maxima. The four maxima come at the following times: *A*, 0·27 sec.; *B*, 0·40 sec.; *C*, 0·46 sec.; *D*, 0·56 sec.

set to the same value and the other ordinates adjusted to correspond. In this way the effects of these various treatments upon the persistence of action potential are more clearly shown.

E. *Summation.*

It appears certain from the results of other workers that, so far as the initial electrical discharge at high voltage is concerned, a second action potential wave does not, and presumably cannot, appear until this phase of the first wave is over. In these initial phases of the two waves, therefore, neither summation nor interference is possible. The situation with regard to the later phases of the wave here discussed is very different. A second wave generated within a short interval will encounter persistent remnants of the first. It is a matter of prime importance to determine whether the retentions connected with the second

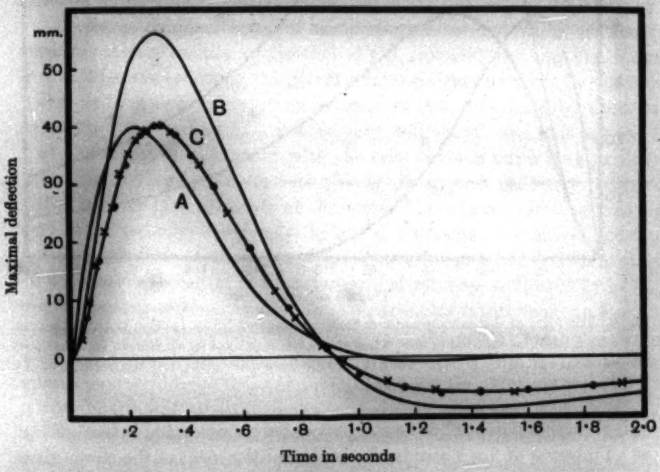

Fig. 5. The form of the galvanometer deflection produced by two action potential waves, with a stimulus interval of 9σ (Curve *B*), compared with that given by a single wave (Curve *C*), and with a physical control (Curve *A*). The experimental points by which Curve *C* is determined are shown (●), as well as the values calculated for Curve *B* (X), when it is reduced to the same maximum ordinate as Curve *C*. The two sets of values fall upon the same curve. Temperature 18° C. ×1 (for ordinates).

These records were secured from the same nerve which gave the curve of Fig. 1. The control curve here shown applies to that figure as well, and shows that the "after-positivity" cannot arise from under-damping.

wave will be added to those already present as elements of the first. It would also be desirable to know whether summation may occur between the initial phase of the second wave and the retentions of the first, but this determination cannot be made by our method.

Experimental results bearing upon the question of summation are presented in Figs. 5 and 6. In Fig. 5 are given the curves produced by

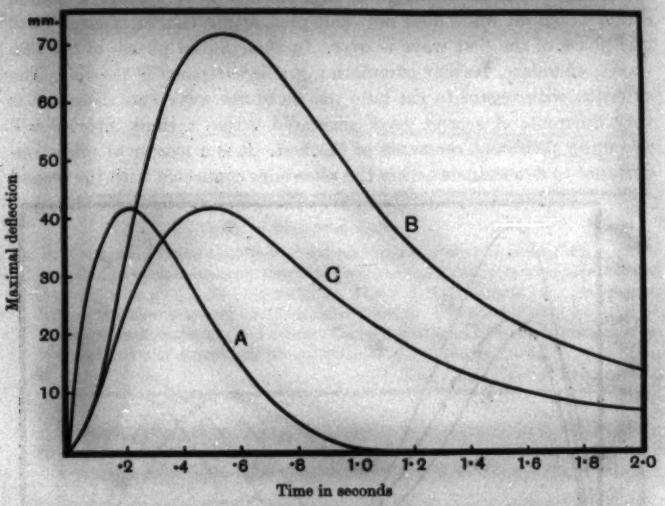

Fig. 6. The form of the galvanometer deflection produced by two action potential waves, with a stimulus interval of 90σ, when the nerve is in expired air (Curve *B*), compared with that given by a single wave (Curve *C*), and with a physical control (Curve *A*). Temperature 18° C. ×1 (for ordinates).

a single action potential wave and by two waves generated by two stimuli at an interval of 9σ. There is also indicated the form of the curve given by the two waves when the maximum ordinate is made identical with that given by the single wave. It is at once evident that there is an almost complete coincidence in form between the two curves.

So complete a coincidence was not to be expected, and we are still unable to give a fully satisfactory explanation of the observation. It seems clear, however, that if the second wave abruptly terminated the first, eliminating all trace of the persistent potentials which would other-

wise follow it, the combined electrical discharge should give a form of galvanometer movement different from that associated with a single wave. The presence of two initial discharges at high voltage combined with the retentions of one wave alone should throw the galvanometer more quickly to its maximum deflection, *i.e.* more nearly approximate the form of the control curve. Since this is not obtained we infer that the second wave does not terminate the first completely. It appears to combine with it in such a manner that the ratio of distribution of potential between initial and delayed phases is not greatly altered.

A complicating factor in such a determination is the presence of an after-positivity, which is certainly summated, as the curves presented in Fig. 5 show. The presence of this phase tends to limit the movement of the galvanometer in its first swing, as we have previously seen, and may be largely responsible for the coincidence between curves given by one and two action potential waves.

A clearer demonstration of summation is secured when we study records taken after the application of CO_2 (expired air). In Fig. 6 are presented curves showing the galvanometer deflections given by a single wave, and by two waves at an interval of 90σ. The long retention of negativity, induced by CO_2, is here very well shown, and it is apparent that the retention associated with the two waves is much greater than that given by one. We are here able to distinguish definitely between initial and delayed elements of the wave. The control curve returns to zero at 1·1 seconds. Obviously the initial discharges at high voltage can have no influence on the galvanometer after 1·2 seconds, and from then on we observe the effect of the retentions alone, uncomplicated by either initial phase or after-positivity. In this particular experiment summation appears to become nearly complete toward the end of the wave, since after 1·2 seconds the ordinates of the curve given by two waves are approximately double those of that given by one.

Summation of retention E.M.F.'s, therefore, is clearly evident in the after-positivity, which is a part of the action potential wave when the nerve is in air, and in the prolonged negativity developed in the presence of CO_2. Less clearly it is seen that the negative retentions in air are probably summated in part. The possible physiological rôle of such summation will be later discussed.

Discussion.

The effects here described are, in large part, not strictly new observations. We believe that the method which we have used is especially

well adapted for the demonstration of such effects, and that our records give new evidence of a convincing sort for their existence. But the older literature contains many studies which relate to the same or similar phenomena. We must now consider that literature and attempt to evaluate our own studies in its light.

An "after-positivity," appearing in nerve at the end of a period of tetanic stimulation was early observed by Hering(7) and has since been much studied, most recently by Woronzow(8) and Gerard(9) who give references to the literature. A few attempts have been made to determine whether such a positive phase is present in a single action potential wave. Head(10) was unable to detect it, but Garten(4) later secured capillary electrometer records from the olfactory nerve of the pike which show it faintly developed. Our records confirm this observation and demonstrate its presence beyond any possibility of doubt. In air and above 15° C. it appears to constitute a normal element of the action potential wave.

The disappearance of the after-positivity which follows a tetanus at temperatures below 15° C. has been observed by Garten(11) and confirmed by Woronzow(8). The former found that the temperature of the killed region may be raised and lowered without affecting the character of the after-positivity, whereas temperature changes produced in the intact region have a great effect upon it; he concluded that the phenomenon is produced in the intact membranes. While Woronzow is unwilling to accept this interpretation we feel that Garten's evidence constitutes a fairly definite proof of his thesis, and increases the probability that, in the after-positivity, we are dealing with a normal physiological effect. Its occurrence in the single action potential wave renders improbable the view that it arises from some abnormal modification of the tissue in the course of a multiple stimulation. It may also be argued that the diminution or disappearance of the retentions when the tissue has been long inactive, their reappearance with activity and their progressive modification under conditions which at least simulate functional states, all point to a normal physiological character.

Waller(1) has argued that the increase in magnitude of the electric response which he observed after a period of multiple stimulation is evidence for the production of CO_2 by nerve during activity, since the administration of CO_2 has a similar effect upon the magnitude of the response. Our records show that the two effects are not identical. In both cases a marked increase in maximal deflection is indeed obtained,

but the curve taken after multiple stimulation shows a well-developed after-positivity, whereas in CO_2 all trace of this element of the action potential wave disappears. The difference between the two types of records is clearly shown in Figs. 3 and 4.

Persistent negativities, other than those induced by CO_2, have also been described, most recently by Levin(12) who gives an excellent summary of some of the older work. In his material, the nerves of the spider crab, the persistent negativities are very highly developed, and cannot escape attention. On the whole, however, it would appear that the tendency among workers in this field has been to ignore such effects or to dismiss them as unphysiological. Thus, in a few experiments done in December and January, Forbes and Thatcher(6) observed string galvanometer deflections produced by single impulses in the frog's sciatic nerve which persisted for as much as 0·1 second, but could find no such retention in records taken in March. They attribute this change to some seasonal variation in the tissue. Davis and Brunswick(13) have occasionally observed such retentions but believe them due to "some undetected error of technique" and dismiss the claim of Broemser(14) that a small secondary wave follows the initial electrical discharge. In addition to the more obvious secondary wave whose presence he stresses, Broemser's records give convincing evidence of a persistent retention of negativity at a voltage so low that he apparently considers it of no importance, and does not mention it. A search through the literature shows other similar cases where negative retentions at low voltage are present. They are clearly evident in the records obtained by the new oscillograph of Mathews(15), and at least a hint of them is found in the records of Rosenberg(16). Bishop(17) using the cathode ray oscillograph has described a persistence of negativity at low voltage enduring for at least 10σ; he believes that this persistent potential may not be a part of the excitation proper, but may be the electrical sign of some recovery process. The results obtained by Gerard and Forbes(18) may also fall in the same class, although their effects disappear with activity, whereas those which we have here described are markedly increased in active states.

These various reports have little in common, save only that secondary waves, or prolongations of negativity of one sort or another are evident. We are not prepared to decide which of these observations present the normal physiological picture, if, indeed, any of them do. With all their diversity, however, they do furnish a background of probability in favour of the view that retentions of negativity are often to be expected in

vertebrate nerve, although the conditions necessary for their production remain obscure. Our own results may, we hope, have the virtue of again directing attention to this phase of action potential study and of increasing the probability that such persistent E.M.F.'s are normal components of the action potential wave.

Unfortunately we are not yet able to make definite statements concerning the absolute magnitude of these persistent effects. We had hoped to be able, by an adaptation of the method of curve analysis devised by Hill and Hartree (19) for their myothermic studies, to secure at least a rough analysis of the form and time relations of the action potential wave as recorded by our method. We have made several attempts to do such analysis, but are not yet sufficiently satisfied with our results to be willing to communicate them. It has always been recognized that this method is not well adapted for the study of physical events in which rapid or extreme changes in magnitude are encountered. There is no doubt that the persistent voltages here described are very small as compared with the electrical discharge at high voltage which initiates the action potential wave. We cannot yet be certain that, in analysis, we can properly allow for this sudden change in order of magnitude. In addition difficulties of another sort arise in the manipulation of diphasic curves, whose analysis is much more uncertain than when monophasic curves are being studied.

We have here given, as control curves, the form of the galvanometer deflection produced by break induction shocks, since the presence of delayed components in the action potential wave can be most clearly demonstrated qualitatively when such curves are used. We might point out, in passing, that a quantitative analysis of our unknown curves cannot be made with such controls. The reasons for this statement and the proper procedure for such an analysis will be described in a later communication.

We must now consider the possibility that the effects here described are in some way due to the fact that we are recording the action potential rather close (10–12 mm.) to the killed end of the nerve. We have deliberately chosen so to work in order to avoid all possibility that diphasic curves of the type described by Bishop, Erlanger, and Gasser (20) could be present to complicate our results, even though we recognize that the magnitude of the nerve impulse is reduced in a region so near to the killed end of the nerve (13). In this region, where a condition exists which is in some respects similar to catelectrotonus, we have good reason to expect that the duration of the action potential

wave will be shortened and its amplitude diminished in a manner altogether similar to that described by Bishop and Erlanger(21). When, in such a region, we observe persistent electromotive effects, these must arise in spite of this condition, rather than because of it. We are prepared to believe that in normal nerve the effects here described are of even greater magnitude than can be observed under our experimental conditions.

In any case there is no resemblance between the triphasic curve of Fig. 1 and the diphasic curve which the oscillograph records when the distance between proximal lead-off electrode and killed region is sufficiently increased. In our case we are dealing with seconds of time; in the other case we have to do with a few sigmata only. The two effects cannot possibly be related.

It is a commonplace of modern neurophysiology that the nerve impulse exhibits an "all-or-none" character. When, therefore, we speak of summation in connection with the phenomena here discussed it becomes necessary to consider the implications of the term. Our evidence shows quite clearly that the retentions may be summated, at least in part. Since this is possible it might at first appear as if we had to do with a situation in which the "all-or-none" law could not strictly hold. However, it appears to be true that the character of the combined wave is independent of the strength of either the first or second stimulus, provided both are maximal. It is possible, therefore, to take the view that the first wave, by virtue of its persistent elements, achieves a temporary modification of the tissue, in which state an "all-or-none" response is obtained by a second stimulus, with a further modification of the tissue. While we here consider only the electrical sign of this modification, it must, of course, be associated with some more complex physico-chemical mechanism.

The interpretation is complicated by the fact that, since, at the shorter intervals, the maximal deflection of the galvanometer is not doubled, summation must be partial only; a certain amount of interference between the two waves is indicated. Here again, however, we may take the view that the first wave is unaltered by the second, but itself permits only a partial development of the retentions in the second wave—an alternative conception which is experimentally indistinguishable from the first. If this alternative is correct we are presented with a situation somewhat analogous to that long known to hold for the initial electrical discharge in the classical relative refractory period. The long delay in recovery considered in our preceding paper might then properly

be described as indicating a relative refractory period—that of the recovery of retention E.M.F.—following a curve with very different time relations from that known to hold for the recovery of the initial high-voltage phase.

It seems to us to be reasonably certain that the long delay in recovery of action potential first described by Zotterman(22) and discussed by us in the preceding paper, results from interference between the second action potential wave and persistent remnants of the first. This view is strengthened by certain definite correlations between the results discussed in the preceding paper and those here presented. Thus the duration of the negative retention in air, as measured by the increase in time to maximal deflection of the galvanometer, is usually quite similar to the delay in recovery of electric response to two stimuli, described in our preceding paper. The effect of CO_2 is to prolong the duration of the negative retention and to increase the delay in recovery of electric response. Below 15° C. the after-positivity disappears. The diminution and final disappearance of this element of the wave is probably responsible for the increase in maximal deflection which, as described in our preceding paper, occurs as the temperature is lowered. In the studies there described we used a more sensitive instrument with a period which may have been long enough to permit the development of the after-positivity before maximal deflection was attained, with a consequent diminution in the magnitude of the galvanometer movement at the higher temperatures. We believe that the elimination of the after-positivity may also be responsible for the fact that in their later courses the recovery curves at 15° and 10° C. are nearly identical. On the whole there appears to be at least a rough correspondence between the two groups of observations, which, therefore, in a sense support each other. A more exact description of the manner in which two waves interfere with each other, or are in part summated, waits upon the development of an adequate method of curve analysis.

The fuller recognition of these later phases of the action potential wave may, we believe, lead to important modifications in our views concerning many phenomena in both peripheral and central nervous systems. It is not our purpose to discuss this aspect of the problem in any detail. But a whole category of phenomena come to mind which may conceivably find an interpretation through the recognition of such persistent electromotive effects as we have here described. Thus Adrian(23) and Cooper(24) have shown that the occurrence of a supernormal phase in the recovery of nerve is intimately related to the *p*H of the tissue, being

produced by the application of solutions more acid than *p*H 7·4 or by the administration of CO_2. This long persistence of an increased excitability occurs under precisely those conditions which magnify the retention of negativity. As Fröhlich (25) has suggested, this persistence of a definite potential in the same direction as that observed in the initial part of the action potential wave may possibly facilitate a second stimulation over a considerable period. Again it is conceivable that phenomena of "summation" and "facilitation" in reflex actions are dependent upon the building up of summated retentions which similarly render the membranes involved more easily excitable. A delicate control of such summations may be exercised by the *p*H of blood or of the tissues immediately surrounding the nerve, so that even such complex functions as the regulation of respiration may possibly be related to such effects. The conception may thus suggest a definite physical mechanism for phenomena which now lack any such foundation. All such suggestions must, of course, remain highly hypothetical for the present. We hope to be able to push the analysis further, and to put the study, if possible, upon a quantitative basis.

Summary.

Photographic records of the deflection of a highly sensitive moving magnet galvanometer, produced by single action potential waves in the sciatic nerve of the frog, or by two waves at short intervals, demonstrate the existence of low-voltage elements in the wave which may persist for seconds.

In air and above 15° C. these elements consist in (1) a retention of negativity, succeeded after 0·5 to 1 second by (2) an after-positivity, enduring for 3 to 5 seconds, which may finally be followed, in the best records, by (3) a more faintly developed terminal negative phase. The voltages involved are low; in the after-positivity they do not rise above 40 microvolts.

In the presence of low tensions of CO_2 (*e.g.* expired air) the after-positivity disappears, to be replaced by a retention of negativity enduring for seconds.

After long inactivity the retentions are much diminished or entirely absent. Upon re-activation of the nerve they appear again, and become the more powerfully developed, the greater the preceding activity.

When a second action potential wave follows the first at a short interval the persistent elements of the two waves are summated, but in part only. This partial summation probably accounts for the long

delay in the recovery of electric response to two stimuli, first described by Zotterman, and further considered in our preceding paper. The second wave probably interferes with the persistent elements of the first, with a consequent diminution in their combined discharge.

We wish to express our appreciation of the support given to this work by Prof. A. V. Hill, and by Prof. C. Lovatt Evans, to both of whom we are indebted for many helpful suggestions and advice.

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STUDIES ON WATER DIURESIS.

Part I. The effect of decerebration, anæsthesia and morphia upon water diuresis.

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Introduction.

In a recent paper the author(2) called attention to the fact that both anæsthetics and morphia prevent the kidney from eliminating any excess water which has been absorbed from the alimentary canal. It is well known that as little as 250 c.c. of water will, in the unanæsthetized dog, rapidly increase the urinary output 20 to 30 times and that this augmented flow is maintained until the excess water is eliminated. During anæsthesia, and after morphia, as much as 1400 c.c. of water may be absorbed from the alimentary canal without causing more than a delayed and transient diuresis, and even during this diuresis the maximum rate of urine flow is seldom greater than eight times the normal. It was suggested that this slight diuresis resulted from an increased rate of glomerular transudation due to definite dilution of the plasma proteins and that anæsthesia interfered with the mechanism normally responsible for water diuresis.

Scott and Loucks(5) sought to avoid the effect of anæsthesia upon urine formation by using decerebrate dogs, but found that the urine flow was greatly reduced for as long as 7 hours after the operation. Their decerebrations were carried out under morphia and chloroform-ether anæsthesia. In view of the obvious advantages of an experimental preparation in which typical water diuresis could be elicited, it was thought advisable to investigate to what extent the use of morphia was responsible for the reduction of urine flow observed by these workers.

Experimental technique.

Dogs only were used in the following experiments. Morphia was not injected unless so stated, and any operative procedure necessary was

performed under chloroform-ether anæsthesia. Decerebration (or decortication) was effected in the usual manner through a trephine hole in the side of the skull; both carotid arteries having been previously ligatured, and a tracheal cannula inserted. Provided that care is taken to avoid undue injury to the circle of Willis and that a piece of fresh muscle is placed upon the cut surface of the brain, such animals will remain in good condition for upwards of 16 hours, and without undue loss of blood. Ureteral cannulæ were then inserted extraperitoneally, a stomach tube passed, and the preparation left for at least an hour in order to allow the effects of the anæsthetic to wear off. Throughout the experiment the animal was kept warm and, in order to avoid reflex movements, was disturbed as little as possible.

Results.

Twelve experiments lasting from 4 to 17 hours are discussed in this paper. Of these, five were performed on decorticated preparations without morphia, four on decerebrate preparations without morphia, and three on decerebrate preparations which had received, subcutaneously, from 10 to 20 mg. of morphine acetate just before the operation. All of the results recorded have since been confirmed by the use of similar preparations for further investigations on water diuresis.

In Fig. 1 the urine flow in five of these experiments, during the first 2 hours after giving water, is compared with the onset of water diuresis in an intact unanæsthetized animal. It is obvious that there is no appreciable difference between the water diuresis in the normal animal and that obtained in decerebrate and decorticate preparations, except in the case where morphia had been injected previous to the operation. If this has been done diuresis fails to take place.

The following experiment illustrates several of the more important factors to be considered:

No morphia was given before the operation, and decerebration was performed under chloroform-ether anæsthesia. An hour and a quarter after the completion of the operation, 900 c.c. of warm water were given by stomach tube over a period of 3 hours. Since the urine flow did not rise above 3·6 c.c. per 15 minutes during this period a midline incision was made in the abdomen, and the stomach contents examined. It was found that none of the water given had left the stomach. 200 c.c. of warm water were then injected directly into the duodenum through a large hypodermic needle. The urine flow immediately began to increase, and at the end of 2 hours had risen from 1·5 c.c. to 19·2 c.c. per 15 minutes. 20 mg. of morphia acetate were then injected intravenously and the urine output at once fell to below 0·5 c.c. per 15 minutes, remaining at this level for 2 hours. 10 c.c. of 30 p.c. sodium chloride was then injected, with the result that during the next 10 minutes

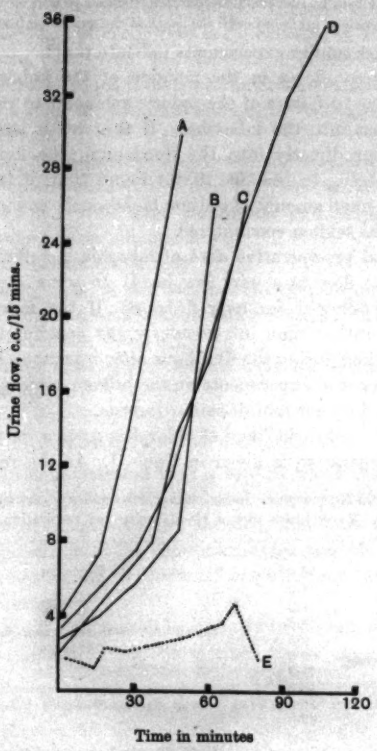

Fig. 1.

A. 8 kilos, ♀. In Pavlov stand. No morphia or anæsthetics. 500 c.c. water by stomach tube. Urine from bladder catheter.

B. 8 kilos, ♂. No morphia. Decerebrated under *C* and *E*. 500 c.c. water by stomach tube. Urine from extraperitoneal ureteral cannulæ.

C. ? kilos, ♀. No morphia. Decerebrated under *C* and *E*. 250 c.c. water direct into duodenum. Urine from extraperitoneal cannulæ.

D. 8·5 kilos, ♀. No morphia. Decorticated under *C* and *E*. 900 c.c. water by stomach tube. Urine from extraperitoneal cannulæ.

E. 7 kilos, ♀. 15 mg. morphia acetate approx. 1 hour before decerebration. 250 c.c. water directly into duodenum. Urine from extraperitoneal cannulæ.

the urine output had risen to 19·0 c.c. This (dilution) diuresis passed off within 20 minutes and the urine flow remained inhibited until the death of the animal an hour and a half later.

This and other similar experiments indicate that:

(*a*) An apparent delay in the reaction of the kidney to ingested water may be due to failure of the pyloric sphincter to relax and allow the water to pass into the intestines. If this factor is eliminated by placing the water directly into the duodenum, the kidney responds almost immediately. In practice it was found that, if fasting animals were used and a small amount of sodium bicarbonate added to the water, this difficulty was seldom encountered.

(*b*) The usual pre-operative dose of morphia is sufficient to reduce the rate of urine flow to a very low level. In some experiments this delay has been followed for over 6 hours. If the morphia is given subcutaneously rather than intravenously the antidiuretic effect does not become marked during the first hour after injection. This delay was previously observed in experiments on the human subject (2).

(*c*) Morphia does not inhibit saline diuresis.

The effect of "pituitrin" and of chloralose upon a water diuresis in a decorticated preparation is given in Table I. As the result of giving

TABLE I. 8·5 kilos, ♀. No morphia. Decortication done under *C* and *E*. Operation completed at 10.30. No excessive loss of blood; pulse and respiration good throughout experiment.

Time	Urine flow c.c./15 mins.	Remarks
11.0 –11.50	1·4	
11.50–12.20	1·0	
12.20–13.0	1·6	
13.0 –13.30	3·5	12.0–13.50. 900 c.c. warm water given through stomach tube
13.30–14.0	9·0	
14.0 –14.15	11·7	
14.15–14.30	18·5	
14.30–15.0	26·5	
15.0 –15.15	32·0	
15.15–15.45	36·0	
		15.45. 10 units "pituitrin" intravenously: anuria till 15.50
15.50–16.20	7·7	
16.20–20.50	2·5	
20.50–23.50	1·5	
23.50– 1.40	7·1	
1.40– 2.25	14·5	
2.25– 3.05	21·5	
		3.05. 0·8 g. chloralose intravenously: anuria till 3.25
3.25– 3.40	4·0	

900 c.c. of tap water by stomach tube the urine flow rose from 1·1 to 36·0 c.c. per 15 minutes. Ten units of Parke Davis "pituitrin" was then given intravenously resulting in a profound drop in the rate of excretion

which lasted for upwards of 7 hours. Diuresis then gradually returned until 22·0 c.c. of urine were being excreted in 15 minutes. 0·8 g. of chloralose was then given, and this once more reduced the urine flow to a very low level at which it remained until the animal died of hydræmic plethora 1 hour after the administration of the chloralose and 16½ hours after the operation.

Respiration, blood-pressure and heart rate remained satisfactory and practically constant until just before the death of the animal. Urine secretion continued even after respiration had stopped, until heart failure supervened a few minutes later.

This experiment indicates that:

(*a*) "Pituitrin" has, in the decorticated preparation, a definite and prolonged antidiuretic action upon water diuresis. (This has also been obtained in decerebrate animals.)

(*b*) Chloralose markedly interferes with the elimination of excess water by the kidney.

Discussion and conclusions.

It has been demonstrated that a typical water diuresis can be obtained in decerebrated and decorticated dogs, provided that morphia and non-volatile anæsthetics are avoided, and that sufficient time is allowed for the effects of the chloroform-ether anæsthesia necessary for the operation to wear off. "Pituitrin" has a profound antidiuretic action upon such a diuresis.

The failure of Scott and Loucks(5) to obtain satisfactory urine flows after decerebration may be ascribed to the use of morphia before the operation.

Molitor and Pick(3) claim that "pituitrin" has but little, if any, antidiuretic effect after decerebration, and Craig(1) obtained similar results in experiments under anæsthesia. Knowlton and Silverman(4) obtained a slight diuresis after giving "pituitrin" under anæsthesia. As has already been indicated, the slight diuresis resulting from the ingestion of water during anæsthesia is of an entirely different nature from that which occurs in the normal animal. Under these circumstances the presso raction of "pituitrin" with the consequent increase of renal blood-pressure might be quite sufficient to account for these observations.

The nature of the inhibitory action of anæsthetics and of morphia upon renal excretion will be discussed in a future communication.

The expenses of this research were defrayed by a grant from the Royal Society.

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A PHOTOGRAPHIC METHOD FOR THE ESTIMATION OF COLOURED SOLUTIONS WITH SPECIAL REFERENCE TO VISUAL PURPLE.

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A method of estimating visual purple or any other light-sensitive substance must satisfy certain conditions over and above those necessary for light-stable substances. Readings must be taken without too long an exposure to any but very dim lights and, for visual purple, the method must be suitable for use with small quantities of solution. Garten's(1) method, which consists in comparing the freshly dissected retina with a standard colour chart, must obviously be inaccurate besides being quite unsuitable for animals such as frogs or coloured rats where it is practically impossible to remove the retina free from pigment epithelium. Hecht(2) used a micro-colorimeter and claims a 5 p.c. error for the estimation of the amount of visual purple present at different stages of bleaching. Since a part of the unbleached solution is used as the comparison, the method is not applicable to the determination of the amount of visual purple in different batches of animals. In both methods the preparation is exposed to a fairly bright light for a variable time.

We used an arrangement whereby an Ilford neutral-tint wedge and an optical cell containing the visual purple solution were photographed simultaneously on the same plate, and the densities of the images were compared. The intensity of the light was not high enough to cause observable bleaching of the solution even after two hours' exposure. As ten minutes only was necessary for each exposure, a large number of readings could be taken on any given specimen. Small quantities of coloured solutions (about 0·5 c.c.) can be economically estimated by this method, which also provides a permanent record. Although devised for small quantities of solution, the method was found to be unexpectedly accurate.

[1] Working with a grant from the Medical Research Council.

Apparatus. Different emulsions, used for making plates, have varying colour sensitivities, and so the relative densities of the photographic images from a coloured solution and neutral wedge will not be constant among different plates unless the light used is approximately monochromatic. Since the solutions were not deeply coloured, we used a green light (Wratten colour filter 61, maximum transmission at 530 $\mu\mu$) approximately coinciding with the maximum absorption of visual purple. The arrangement of the apparatus will be clear from the diagram.

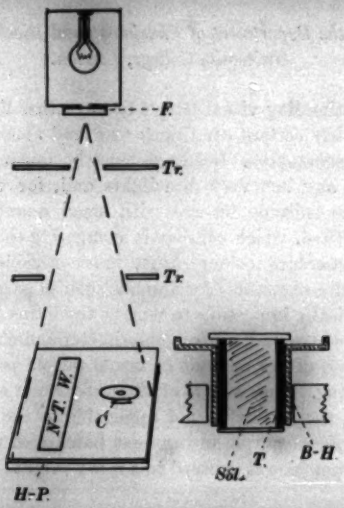

Fig. 1. Diagram of the apparatus and of the optical cell. *F.* Colour filter. *Tr.* Light traps. *N.-T.W.* Graduated neutral-tint wedge. *C*, Optical cell. *H.-P.* Photographic plate in holder. *Sol.* Solution to be measured. *T.* Black glass tubing with clear glass cemented on bottom. *B-H.* Brass casing and collar.

Method. Ilford rapid chromatic backed plates were found to be suitable. They were exposed and developed (in M-Q) for a standard time. Although the time of exposure and development cannot influence the relative densities of the wedge and cell images, it is convenient to have an image which is neither too light nor too dark. The negatives were printed on glossy Slogas paper and the image of the cell was cut out, placed in a holder and slid along the image of the wedge to find the point of equal density. The distance of this point from the edge of

the wedge was measured. Owing to the strong contrast of the print this point was easy to find.

Calibration of wedge. The wedge was calibrated by making a series of exposures in which the cell was replaced by standard Wratten neutral-tint filters[1]. The point of equal image density was found for each filter. The calibration was carried out at six different points on the wedge and five plates were exposed for each point. Three prints were then taken off each negative and the values averaged. A representative set of readings for the filter whose density was 0·592 is given in Table I.

TABLE I. Sample of readings taken during the calibration of the wedge. Density of filter: 0·592. Plates: Ilford rapid chromatic (backed).

Plate number	39	40	41	42	43
Readings from 3 prints in cm.	2·38	2·31	2·49	2·34	2·42
	2·43	2·28	2·42	2·33	2·43
	2·40	2·31	2·47	2·26	2·40
Mean for plate	2·40	2·30	2·46	2·31	2·42
Mean for filter and "probable error"			$2·38 \pm 0·02$		

Another set on Hauff analo-flavine plates gave a mean of $2·40 \pm 0·09$ with the same filter.

It was found that although a set of prints off one negative gave very constant readings, there was more variation between the values for different negatives. Thus it would appear that the printing paper was less variable than the plates.

When a graph was drawn relating the density of the wedge with the distance from its end, it was found that five out of the six points fell on a straight line. The sixth point, which was very close to the thin edge of the wedge, fell a little off the line. For the greater part of its length, the wedge was found to change 0·208 in density for each centimetre of its length. In the following list the distance along the wedge of each photometrically obtained point is given, followed, in brackets, by the distance along the wedge calculated from the equation of the best fitting straight line: 7·42 (7·45); 5·38 (5·39); 3·91 (3·90); 2·38 (2·33); 0·90 (0·95) cm.

If this method is to be used for the estimation of ordinary coloured solutions such as potassium permanganate, a slight correction must be applied for the absorption of light by the cell used. This correction is

[1] These filters were calibrated by the National Physical Laboratory. The density of a filter or solution is expressed by the logarithm of the reciprocal of the fraction of the light transmitted. Thus, a filter transmitting 1 p.c. (0·01) of the incident light has a density of $\log\left(\frac{1}{0·01}\right)=2$. In this notation the concentration of a solution (grm. per litre) is directly proportional to its density.

not necessary when estimating visual purple, since the true absorption is obtained by a difference method—by subtracting the value of the bleached from that of the unbleached specimen. To find the cell correction we took a set of exposures with the cell in position, filled with water and with a neutral-tint filter on top of it. By this method we found that the cell had a density of 0·083, and this is equivalent to a distance of 0·4 cm. on the wedge, to be subtracted from each reading.

We took one series of readings for potassium permanganate solutions of three known concentrations. The calculation of the concentration of such a solution from the values obtained will be clear from Table II.

TABLE II. Determination of the concentration of two solutions of $KMnO_4$, given the concentration of a third solution (0·0790 grm. per litre). "Probable errors" of measurements shown in brackets.

Five plates were exposed for each concentration.

Actual concentration of solutions, grm. per litre *a*	Point of equal photographic density in cm. from end of wedge *b*	Readings in column *b* with correction of 0·4 cm. for cell density *c*	Equivalent density on neutral-tint scale *d*	Concentration calculated from photometric measurement = column $d \times k$* (*k* constant) grm. per litre *e*	Error = difference between columns *a* and *e* grm. per litre *f*
0·1053	7·30 cm. (0·06)	6·90 cm.	1·5320	0·1042 (0·0008)	0·0011
0·0790	5·52 „ (0·03)	5·12 „	1·1617	0·0790 (0·0004)	—
0·395	2·76 „ (0·02)	2·36 „	0·5876	0·399 (0·0003)	0·004

* $k = \frac{0{\cdot}0790 \text{ (column } a\text{)}}{1{\cdot}1617 \text{ (column } d\text{)}} = 0{\cdot}068.$

SUMMARY.

1. A photographic method satisfying the conditions necessary for the estimation of visual purple is described.

2. An approximately monochromatic light is simultaneously passed both through the solution to be estimated and through a graduated neutral-tint wedge, onto a photographic plate. The densities of the images are compared.

We wish to thank the Medical Research Council (Physiology of Vision Committee) whose help made this investigation possible.

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THE COMBINATION OF CARBON DIOXIDE WITH MUSCLE: ITS HEAT OF NEUTRALIZATION AND ITS DISSOCIATION CURVE.

By G. STELLA (*Padua*)[1].

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The anaerobic formation of 1 g. of lactic acid in muscle is accompanied by a liberation of heat which is estimated by Meyerhof and Suranyi(1) as 380 to 390, say 385, calories. The origin of the whole of this heat is still not clear. According to these authors about 180 calories, according to Slater(2) 235 calories, are due to the formation of dilute dissolved lactic acid from hydrated glycogen, as calculated from the difference between the heats of combustion and from the heats of dilution and hydration. There remain 205 calories, or 150 calories, respectively, to be explained. Meyerhof(3, 4) has found the heat of acid dissociation of muscle proteins to be very high, namely about $-12{,}600$ calories per g. molecule, so that if the whole of the lactic acid formed were neutralized by combination with the alkali protein of the muscle, there would be about 140 calories available, an amount practically sufficient to account for the remaining heat if we accept Slater's value for the heat of combustion of glycogen, some 65 calories too little if we accept Meyerhof's value. If, however, an appreciable fraction of the neutralization were at the expense of other buffers, for example K_2HPO_4, the heat of neutralization might be much less, namely for the fraction neutralized in this way about 1700 calories per g. molecule, *i.e.* 19 calories per g. of lactic acid (Meyerhof(3)). It is very necessary, therefore, to ascertain the nature of the reaction by which in fact lactic acid is neutralized in muscle.

Meyerhof and Lohmann(5) by alcoholic extraction of fatigued and unfatigued muscle arrived at the result that of all the base bound by lactic acid formed in muscle only one-half to two-thirds is derived from alkali protein, the remainder originating from other sources such

[1] Fellow of the Rockefeller Foundation.

as phosphate and carbonate. If this were so the neutralization of lactic acid by muscle should give rise only to 80 to 100 calories per g. of acid. It should be noted that bicarbonate cannot play any rôle in this respect unless time be given for the CO_2 formed on combination with acid to escape from the muscle, since the heat of neutralization of CO_2 by muscle is practically the same as that of lactic acid, and therefore if CO_2 be driven off from bicarbonate by lactic acid it merely combines with the alkali protein, thus liberating the same heat as if the lactic acid combined directly.

Since the nature of the alkali protein of the living muscle might very well be altered by chemical treatment and extraction, and since the major portion of the phosphate in muscle has been shown by Eggleton and Eggleton(6) to exist in organic combination and not simply in the inorganic form, the safest method of getting a true figure for the heat of neutralization of lactic acid in muscle is its direct determination. Meyerhof(3) investigated the heat liberated per g. of muscle when the upper legs of a frog were soaked in an approximately equal amount of Ringer's fluid containing valerianic acid in such a concentration as to give, after equi-partition, a concentration of 0·25 to 0·3 p.c. inside the muscle. He found values of about 0·3 calorie per g. of muscle, which would correspond roughly, in the case of lactic acid, to about 100 calories per g. of that acid neutralized. He did not determine, however, the final concentration of the valerianic acid inside the muscles, nor their final *p*H, and the time taken in each experiment for the acid to penetrate the whole muscle was very long—3 or 4 hours—thus rendering the heat measurements rather uncertain. Moreover, some control experiments in which the valerianic acid was changed for a neutral solution of sodium acetate in Ringer's fluid, gave heat about 0·1 calorie per g. of muscle as compared with about 0·3 under the action of the valerianic acid. Thus Meyerhof's experiments do not give any very precise idea of the absolute amount of heat liberated in muscle by the neutralization of lactic acid, though they do show that this is much higher than it would be were the buffers in muscle mainly phosphate and bicarbonate.

The present work represents a new attempt to determine directly the heat of neutralization of lactic acid by living muscle. Instead of a solution of valerianic acid, carbon dioxide gas was used, and instead of the upper legs of a frog the very small and thin sartorius mounted on a sensitive thermopile. The method, which has already been described by A. V. Hill ((7), p. 154), has the advantage, as compared with that of Meyerhof, that the diffusion of the gas may be complete in a few

minutes, owing to the thinness of the muscle, thus rendering the heat measurements more accurate. Moreover, the process is reversible: the carbon dioxide can be removed by a stream of nitrogen or oxygen and the muscles return to their original condition[1]. Hence in experiments on the heat of neutralization of carbon dioxide by muscle we are dealing with the action of living tissues in good condition. As found by Hill in the paper quoted, the carbon dioxide does not cause any appreciable change in the resting heat rate, so that in the deflection-time curve of the galvanometer the heat produced by the CO_2 can easily be separated from that due to resting processes occurring spontaneously in the muscle. The speed of diffusion of CO_2 is relatively high, and the measurement of the heat can be completed in a few minutes. Afterwards, if desired, the CO_2 can be removed by nitrogen and negative heat equal to the original positive heat will be found.

When an atmosphere of pure CO_2 is introduced around a muscle there is a rise of temperature due partly to the heat of solution of the gas and partly to the heat of combination of carbonic acid with the alkali available. The latter heat is the sum (*a*) of the negative heat of dissociation of carbonic acid into its ions, which is equal to -2800 calories per g. molecule (Landolt and Börnstein's Tables), and (*b*) of the positive heat of unionization of the buffering acid or acids involved. Thus, for our present purpose we need to know (1) the total heat, (2) the solubility of CO_2 in muscle, and (3) the CO_2 combining capacity of muscle at the temperature and the CO_2 partial pressure of the experiment. The total heat is measured. For the solubility we will assume the figure given by Fenn(9), according to whom a muscle dissolves an amount of CO_2 equal to that dissolved by 80 p.c. of its weight of water. The heat of solution of CO_2 at constant pressure is given by Brown and Hill(10). The experimental determination of the amount of combined CO_2 is described in a later part of this paper. Since in the heat experiments an atmosphere of nitrogen was changed for one of CO_2, the combined CO_2 was determined in both conditions and the difference calculated. The average amount of CO_2 combined in 1 g. of muscle at 740 mm. Hg partial pressure of CO_2 (which represents the average CO_2 partial pressure in the heat experiments) and at an average temperature of 19° C. was found to be 0·47 c.c. In nitrogen the CO_2 combined in the muscle was found to average about 0·02 c.c. per g. The difference, therefore—

[1] After removing the CO_2 the muscles are inexcitable but regain their excitability on soaking in Ringer's solution; the excitability is apparently of the reversible type described by Dulière and Horton(8).

0·45 c.c.—represents the extra amount of CO_2 combined when nitrogen was changed for CO_2.

Experimental. For the heat measurements the myothermic apparatus recently described by A. V. Hill(7) was employed. This apparatus consists of a sensitive thermopile, kept at a temperature constant within 0·001° C., connected to a moving coil galvanometer. It allows a fairly accurate measurement of the heat liberated at rest by a small and thin sartorius. During any process examined the extra heat liberated is given by the area of the deflection-time curve above its resting zero line. English and Dutch *Rana esc.* and *Rana temp.* were used without distinction. They were kept previously at 2° C. for some days. In nearly all the experiments a pair of sartorii was employed. After careful dissection they were mounted on the thermopile and kept in Ringer's solution at *p*H about 7·4, containing phosphate to the amount of 8 mg. p.c. P. The chamber containing thermopile, muscle and Ringer's solution was put into the bath at constant temperature and oxygen was bubbled through the solution in order to maintain the muscles in a good resting condition and to quicken the process of equalizing the temperature of the thermopile with that of the bath. After an hour or two the solution was sucked out and the stream of oxygen changed for one of oxygen-free nitrogen. After 40 to 45 minutes, when the muscles were entirely free from oxygen and the galvanometer had settled down to a constant position representing the resting heat-rate, the experiment was started. If resting muscles were the object of study the carbon dioxide gas was let in at this moment; if fatigued muscles were to be investigated the muscles were stimulated in nitrogen by single maximal break shocks, 42 per minute, and the heat liberated by stimulation recorded and calculated, as described by Hill. From this heat the amount of lactic acid produced per g. of muscle was estimated, assuming that 1 g. of lactic acid corresponds to 385 calories. After fatigue the carbon dioxide was run in as in the previous case.

In most experiments the carbon dioxide, which was carefully prepared free from oxygen, was introduced into the muscle chamber very slowly. In five experiments, however, it was introduced rapidly in order to change as quickly as possible the atmosphere in the chamber and so to allow the gas to act upon the muscles at its maximum partial pressure from the beginning of the diffusion. Most of the experiments were performed in April and May: five, however, in November gave practically the same results.

There is an immediate deflection when the first traces of carbon

dioxide come into the chamber, and this continues until an equilibrium is attained between the muscles and the gas. The experiment occupies 10 to 20 minutes. The area of the deflection-time curve was measured above a line joining the initial to the final steady position of the galvanometer, and the heat production was calculated from the area of this curve according to the method described by Hill(7).

In Table I are shown the results of experiments on resting muscle. The variations between different experiments are obviously comparatively small, allowing us safely to assume an average value of 0·380 calorie per g. of muscle.

TABLE I. Total heat produced by carbon dioxide in resting muscle.

Temperature	Heat by CO_2 per g. of muscle (in calories)	Remarks
18° C.	·360	April and May
17·7° C.	·398	
17·2° C.	·397	
20·0° C.	·407	
20·4° C.	·342	A. V. Hill ((7), p. 154), March
"	·406	
"	·359	
"	·430	
18·7° C.	·373	November
19·5° C.	·321	
19·5° C.	·376	
Mean 19·3° C.	Mean ·379	

Now at 19° C. and 740 mm. Hg partial pressure (atmospheric pressure less water vapour pressure) the amount of CO_2 dissolved in 1 g. of muscle is 0·89 (*i.e.* the solubility coefficient of CO_2 in water) multiplied by 0·8 (*i.e.* Fenn's factor) multiplied by 740/760: result 0·695 c.c. The heat of solution of CO_2 at constant pressure, calculated by interpolation for 19·3° C. from the data in the paper by Brown and Hill(10), is 4820 calories per g. molecule. For 1 g. of muscle, therefore, the heat of solution of the carbon dioxide simply dissolved is $0·695 \times 4820/22,400 = 0·15$ calorie. The remaining $0·38 - 0·15 = 0·23$ calorie represents the heat of solution plus the heat of ionization plus the heat of neutralization of the 0·45 c.c. of carbon dioxide combined, or 11,450 calories per g. molecule. The heat of solution is 4820 calories and the heat of ionization is -2800 calories, so that the heat of neutralization is $11,450 - 4820 + 2800 = 9400$ calories, say. This is 3/4 of the value given by Meyerhof, namely 12,600 calories, for the heat of neutralization by alkali-protein and seems to indicate that the resting muscle neutralizes acid to the extent of 3/4 by the unionization of its alkali-protein buffers, and only to the extent of 1/4 by other buffers. These other buffers are presumably

phosphates, since it is obvious that, in the present experiments with CO_2 and in any experiments in which CO_2 is not able to escape, bicarbonate cannot play any part. It should be noted that one atmosphere of CO_2 makes the muscle approximately as acid as the lactic acid maximum produced by fatigue (see below), so that presumably about 3/4 of the lactic acid liberated by stimulation to extreme fatigue is neutralized in the same way by the use of proteins rather than phosphate. Thus the neutralization of lactic acid in muscle is accompanied by the liberation of about 105 calories per g., *i.e.* a somewhat higher figure than that given by Meyerhof and Suranyi(1), according to whom it is about 80 calories.

From this appropriate determination of the part played by phosphate in the neutralization of carbon dioxide it is possible roughly to calculate its concentration in muscle. If 1/4 of the total combined carbon dioxide, *i.e.* 0·11 c.c. per g. of muscle, combines with phosphate, then 0·15 mg. of phosphate changes from K_2HPO_4 into KH_2PO_4. Now when a muscle in equilibrium with nitrogen is brought into equilibrium with carbon dioxide at a partial pressure of 740 mm. Hg, its *p*H decreases from about 7·2 to about 6. The ratio K_2HPO_4/KH_2PO_4 at *p*H 7·2 is 7/3, at *p*H 6 it is 1/9 (Sörensen's data quoted by Clark(11)). In 1 g. of muscle therefore, according to our calculation, 0·15 mg. of P is transformed from K_2HPO_4 to KH_2PO_4 by a process in which 7/10 of the total P in the form of K_2HPO_4 becomes 1/10. Thus 6/10 of the total P = 0·15 mg., and the total P = 25 mg. per 100 g. This calculation of course is only approximate, but it is interesting to obtain a figure of the same order of magnitude as is found by chemical estimation of the inorganic phosphate.

The investigation of the heat of combination of carbon dioxide in muscle has been extended to muscles containing various amounts of lactic acid as a result of previous stimulation under anaerobic conditions. Two series of experiments were performed, one on the heat by carbon dioxide, with the technique already described, another on the amount of carbon dioxide combined. In both cases the muscles after stimulation in nitrogen for various lengths of time were brought into equilibrium with an atmosphere of CO_2 at a partial pressure of about 740 mm. Hg.

Table II shows the results of the heat experiments. In column 3 instead of the concentration of lactic acid its heat of neutralization is given, calculated by multiplying the observed heat of stimulation by 105/385, which, according to the above results, represents the ratio of the heat of neutralization to the total heat accompanying the anaerobic formation of 1 g. of lactic acid in muscle. Column 6 gives the heat of combination of carbon dioxide per g. of muscle, calculated by subtracting from the total heat by carbon dioxide, the part due to the simple solution of this gas. The latter depends on the temperature; as this varied in the different experiments it seemed safer to make a separate correction for each case, instead of taking the average temperature and

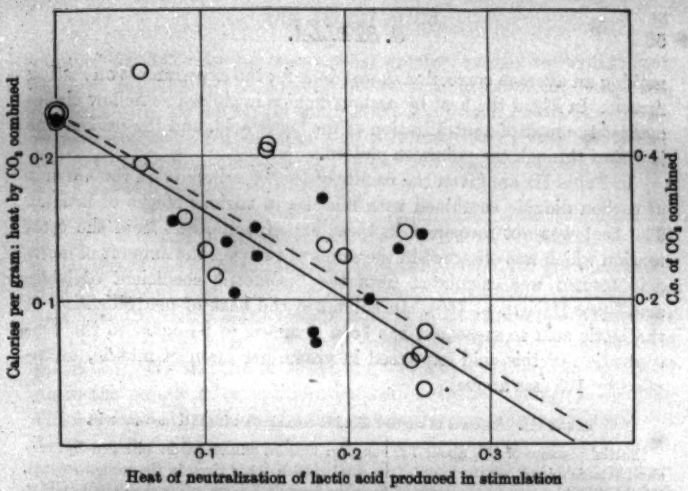

Fig. 1. Hollow circles and broken line: relation between total heat by combined CO_2, when muscles were subjected to 1 atmosphere of that gas, and heat by neutralization of lactic acid in previous anaerobic stimulation (heat calculated from heat observed during stimulation).

Full circles and continuous line: relation between amount of combined CO_2 and heat by neutralization of lactic acid in previous anaerobic stimulation (heat calculated from observed Σ T1 and the "isometric coefficient").

TABLE II. Heat of combination of carbon dioxide with fatigued muscle.

Partial pressure of CO_2 about 740 mm. Hg: muscles stimulated in nitrogen by single shocks, 42 per minute, to the degree indicated by the second column.

Temperature (°C.)	Heat by stimulation (calories per g. of muscle)	Heat by neutralization of lactic acid (calories per g. of muscle)	Heat by total CO_2 (calories per g. of muscle)	Correction for dissolved CO_2 (calories per g. of muscle)	Corrected heat by CO_2 combined (calories per g. of muscle)
17·6	·530	·145	·365	·158	·207
18·6	·214	·058	·348	·155	·196
17·2	·318	·087	·318	·161	·157
17·0	·720	·197	·292	·162	·130
17·5	·397	·108	·376	·158	·117
17·0	·921	·250	·224	·162	·062
17·7	·928	·253	·196	·158	·038
18·0	·372	·101	·290	·155	·135
18·0	·525	·144	·358	·155	·203
19·2	·930	·254	·226	·148	·078
19·7	·214	·058	·405	·147	·258
20·0	·885	·241	·293	·146	·147
19·4	·673	·184	·285	·147	·138
19·2	·897	·245	·205	·147	·058

making an average correction as was done for the experiments on resting muscle. In Fig. 1 the heat by carbon dioxide is plotted as hollow circles against the heat of neutralization of the lactic acid, and the broken line is drawn through the points so plotted.

In Table III are given the results of the experiments on the amount of carbon dioxide combined with muscles in various stages of fatigue. The heat was not measured in these experiments, but from the total tension which was observed in the series of twitches the amount of lactic acid formed was calculated from the "isometric coefficient of lactic acid" (see Hill(7), p. 169). In column 6 the heat of neutralization of the lactic acid so measured has been assumed to be equal to the concentration of this acid expressed in grams per gram of muscle, multiplied by 105 (see above).

TABLE III. Amount of carbon dioxide combined with fatigued muscle.

Partial pressure of CO_2 about 740 mm. Hg: muscles stimulated in nitrogen by very short tetanic stimuli, 42 per minute, the total tension being given in the second column. The lactic acid produced is calculated from the formula 1 g. cm. of tension-length = 10^3 g. of lactic acid.

Temperature (°C.)	Total tension developed (g.)	Muscles		Lactic acid produced per 100 g. of muscle (g.)	Heat of neutralization of lactic acid (cal. per g. of muscle)	Amount of CO_2 combined in 1 g. of muscle (c.c.)
		Length (cm.)	Weight (g.)			
19·0	8,120	3·7	·27	·11	·116	·28
19·0	11,400	3·9	·215	·205	·215	·20
19·0	5,900	3·4	·130	·129	·136	·26
19·0	12,900	3·4	·180	·240	·252	·29
19·2	7,800	3·3	·150	·170	·178	·14
19·1	4,400	3·1	·175	·075	·079	·31
20·0	11,100	3·2	·210	·168	·176	·155
22·0	5,100	2·8	·125	·115	·121	·210
20·5	4,550	2·7	·095	·130	·136	·300
21·0	14,200	3·2	·203	·225	·236	·27
21·5	7,600	2·9	·125	·174	·183	·34

Fig. 1 shows the relation between amount of combined carbon dioxide (full circles) and heat by neutralization of lactic acid, and the continuous line is drawn through the points. A comparison of the points and the two lines in Fig. 1 shows that for a given increase by stimulation in the concentration of lactic acid in muscle, the decrease of the heat by carbon dioxide is, within the limits of experimental error, proportional to the decrease of the amount of combined carbon dioxide; so that the heat by combination of 1 g. molecule of carbon dioxide is the same in a muscle at rest as in a muscle previously stimulated to different degrees of fatigue.

The total heat liberated in the formation of 1 g. of lactic acid in

muscle, viz. 385 calories, is somewhat greater, on any reckoning, than the heat available in the chemical processes known to occur. Accepting Slater's value for the heat of combustion of glycogen, 340 calories should be liberated when 1 g. of lactic acid is formed from glycogen and neutralized: accepting Meyerhof's value, 285 calories. Some chemical process is still required to fill the gap.

The CO_2 dissociation curve of frogs' muscles.

As stated above, in calculating the heat liberated, per gram molecule, by CO_2 reacting with muscle, it is necessary to ascertain the amount of combined CO_2 in the resting tissue (*a*) kept in an atmosphere of nitrogen for some specified time, and (*b*) in equilibrium with an atmosphere of moist CO_2. The method of Warburg was employed, acid being tipped on to the muscle after equilibration, without other change in the conditions. A Barcroft differential manometer apparatus was used, the bottles being slightly modified to allow the muscles to stick on to the wall in a good extended position and so to reach equilibrium more quickly. A side tube with tap permitted the required gas to flow through the bottle, and thence to pass out through the three-way tap of the manometer without contact with the capillary tube of the latter. In the control bottle only a few drops of distilled water were placed, that is to say the arrangement was used differentially only so far as temperature changes were concerned. Since the method gave good and consistent results the experiments were extended to a wide range of CO_2 pressures, allowing the muscles to come into equilibrium with a series of known mixtures of nitrogen and CO_2, the combined CO_2 being determined as before by turning it out with acid. In this way a "CO_2 dissociation curve" of frogs' muscle was constructed.

The experiments were begun in May, 1928, and it was not realized until later that Fenn(9) had been engaged in a similar investigation. Since his method is different, and not entirely free from objection, the present experiments were continued. In principle Fenn's method consists in the sudden replacement of the gas (*e.g.* a CO_2 mixture) with which the tissue has come into equilibrium by another gas (*e.g.* oxygen), followed by the measurement of the CO_2 given out or taken up before a new equilibrium is reached. The replacement of the one gas by the other necessarily occupies a few seconds, and in this time it can be calculated from the diffusion equations (see *e.g.* Hill(12), p. 70) that a considerable fraction, perhaps as large as 15 p.c., of the CO_2 has escaped. Diffusion is extremely rapid in the first moments after such a sudden change in

conditions. In the method of turning out the combined CO_2 by acid no such change of conditions is necessary, and no error of this kind arises[1].

The partial pressures of CO_2 in Fenn's experiments did not exceed 250 mm. Hg. It is interesting to follow the curve to pressures greater than this, for two reasons: (*a*) by extending the range it is possible to determine its later slope more accurately, and (*b*) the higher pressures give us important information of the buffering capacity of muscles in the neighbourhood of *p*H 6·0, *i.e.* of isolated muscles stimulated to exhaustion, or of intact muscles in the living body after extremely violent exercise of short duration where the oxygen debt is high and the amount of lactic acid large. The complete range, therefore, up to one atmosphere of CO_2, has been studied in the present experiments.

Another difference between the present experiments and Fenn's is that the former employed mixtures of CO_2 and nitrogen, the latter mixtures of CO_2 and oxygen. The present experiments in pure nitrogen agree with a few of Fenn's in that gas, in showing only 2 to 3 volumes p.c. of combined CO_2: whereas for pure oxygen Fenn gives a figure of 8 to 10 volumes p.c., which in Fig. 3 below is seen to correspond to a partial pressure of CO_2 in nitrogen of 10 to 15 mm. Hg.

The difference is probably due to two factors: (*a*) the lactic acid formation in nitrogen, tending to displace CO_2 from bicarbonate, and (*b*) the formation of fresh CO_2 in oxygen. Taking the rate of lactic acid formation at 16° C. to be 0·2 mg. per g. per hour, and assuming that one molecule of lactic acid displaces one molecule of CO_2, this would mean a loss of 5 volumes p.c. of CO_2 per hour in a muscle standing in nitrogen. In the present experiments an interval of 45 to 50 minutes was allowed for equilibrium to be attained, so that the total CO_2 combining capacity might be expected to be diminished by 3·7–4·2 volumes p.c. A very rough calculation can be given of the amount of diffusible CO_2 contained in a resting frog's muscle in oxygen at 20° C. owing to the new formation. In a "steady state" (see Hill(12), p. 51), if a c.c. per g. per minute be the rate of formation of CO_2 in a muscle b cm. thick exposed to oxygen on one side only, and if k be the diffusion constant of CO_2, the amount contained, per c.c. of muscle, will be $ab^2/3k$. Taking a as 7×10^{-4}, b as 0·1 cm., and k as $5{\cdot}4 \times 10^{-4}$ (calculated from Krogh(13)), this is 0·43 volume p.c. of diffusible physically dissolved CO_2, which would have an average partial pressure of about 4 mm. Hg. Thus there is an appreciable partial pressure of CO_2 in a muscle resting in pure

[1] Some experiments were performed by Fenn with a direct method, but he does not say how far these results agree with those made with his indirect method.

oxygen, due to its new formation: this pressure, owing to the shape of the dissociation curve, should be sufficient to account for the quantities found by Fenn. These two factors together are probably adequate to explain the difference between Fenn's experiments and those described here: both are affected by an error, and it is difficult to see how this can be completely avoided. The error due to the new formation of CO_2 by a muscle in oxygen is the more serious. One obvious precaution is to employ as thin muscles as possible in order either (*a*) to quicken equilibration in nitrogen, or (*b*) to lower the content of CO_2, due to new formation, in oxygen.

Experimental. As in the above experiments on the heat production, thin sartorius muscles only were employed, a pair for each experiment, weighing on the average 100 to 150 mg. the pair. They were dissected very carefully, cut as near as possible to their connections with the bone, and soaked for one hour in oxygenated Ringer's solution, containing 8 mg. p.c. P, at *p*H 7·3.

The muscles, although perhaps slightly injured owing to their removal from the bones, seemed always in a good resting condition, and never showed any shrinking or spontaneous twitches. Some of them, tested after soaking, were found to be normally responsive to electrical stimuli. The temperature of the room was 16° to 19° C. After an hour in Ringer's solution they were gently and quickly freed from adherent solution by touching with filter paper, weighed on a torsion balance, and placed in the muscle chamber of a Barcroft differential manometer, in a little side pocket *a* (see Fig. 2). At the bottom of the chamber 0·3 c.c. of a 20 p.c. solution of tartaric acid was run in: the chamber was fixed in position and evacuated by a water aspirator, in order to remove from the tissue and the acid solution all the carbon dioxide and oxygen present; a mixture of nitrogen and carbon dioxide in the proportions required was passed through, entering from the side tube *b* and leaving through the tap tube *c*—the gas was allowed to run slowly for 35 to 40 minutes, during which 1 litre of it passed through the bottle; then taps 1 and 2 were closed, putting the chamber into communication with the manometer, and both the bottles of the apparatus were placed in a water bath at 17° to 19° C. By shaking the apparatus and observing the reading of the manometer it was easy to make sure of the moment when the diffusion process had been completed and the acid solution and the muscle had reached equilibrium with the gases in the bottle. The acid was then poured on the muscles by gently tilting the apparatus, and the amount of carbon dioxide driven off determined

and calculated for N.T.P. The time required to drive out the combined carbon dioxide was 30 to 40 minutes, so that it was thought unnecessary

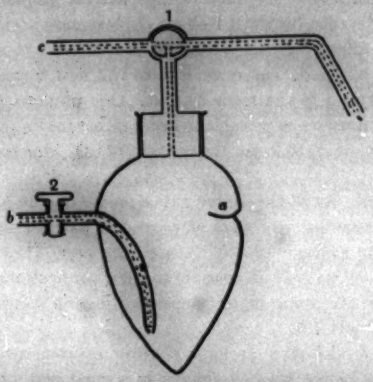

Fig. 2. Muscle chamber of Barcroft differential apparatus: *a*, side pocket for muscle; *b*, side tube for admitting gas; *c*, tap tube, for exit of gas.

to grind up the tissue as Fenn did. For the calibration of the apparatus a method suggested by Professor Barcroft was employed: this consists in introducing into one of the two bottles (in the present case the bottle which served as a muscle chamber) a known volume of air, by means of a mercury pump kept at the same temperature as the apparatus. The displacement in the level of the clove oil in the capillary tube of the latter was found to be directly proportional to the volume of air introduced. This method, compared with that of mixing inside the bottle a solution of Na_2CO_3 with one of acid, has the advantage of being much quicker and of eliminating a possible cause of error due to the presence of bicarbonate in the solution of Na_2CO_3.

The mixture of nitrogen and carbon dioxide was prepared in a bottle above acidulated water in diffusion equilibrium with the gas, by exhausting the bottle and then filling it up with the two gases in the ratio required. Water coming from a second bottle in communication with the first one and in equilibrium with the same partial pressure of carbon dioxide was used to force the gas through the muscle chamber. Two samples of the mixture, one at the beginning and one at the end of the procedure of passing the gas through the muscle chamber, were taken and analysed for carbon dioxide by the Haldane gas analysis apparatus.

In order to know exactly the amount of carbon dioxide combined it was necessary to find out whether all the gas coming out of the tissue was derived from bicarbonate, or if part of it represented CO_2 physically dissolved in the muscles and driven out by their acidification, in other words, to ascertain whether saturation with acid diminished the solubility of carbon dioxide in muscle. Six control experiments therefore were performed, in which the muscles in the chamber (Fig. 2) were replaced by their weight of distilled water. When the acid solution and the water had come into equilibrium with the carbon dioxide in the chamber (740 mm. Hg partial pressure) the acid was mixed with the water and a displacement observed in the manometer, indicating a diminution of 5 p.c. in the solubility of carbon dioxide in water. In all the experiments on muscles, therefore, from the apparent quantity of carbon dioxide combined, 5 p.c. of the amount of that gas physically dissolved at that partial pressure was calculated and subtracted.

When muscles were to be investigated after fatigue, they were taken from the body with their connections to the pelvic bones, connected to an isometric lever in the ordinary way and immersed in the phosphate Ringer's solution. After two hours the Ringer's solution was sucked out and nitrogen passed through the chamber for 40 to 45 minutes; then the muscles were stimulated in nitrogen by very short tetanic stimuli (42 per minute) for different lengths of time and until fatigue had developed. Carbon dioxide was at this moment substituted for nitrogen, and after 15 minutes the muscles were rapidly cut off from the bones, weighed and put into the Barcroft's bottle full of CO_2, the remaining procedure being just the same as for the resting muscles. The rapidity of these last manipulations was necessary in order, so far as possible, to prevent the tissues from coming into contact with the oxygen of the air with consequent aerobic disappearance of some of the lactic acid formed during stimulation.

Some experiments in rigor were performed; the sartorii were dissected, soaked in Ringer's solution for one hour, then weighed and placed in the Barcroft's bottle as before. Nitrogen was passed for 45 minutes to allow all the oxygen to be turned out, the bottle was then closed and placed in a water bath at 40° C. for one hour, until full rigor had developed. Then carbon dioxide was introduced as described above. By this procedure any contact with oxygen of the muscles in rigor was avoided.

The results of the experiments on fatigued muscles have been reported above; on rigor muscles three satisfactory experiments were

made at a partial pressure of about 745 mm. Hg, giving a concentration of combined carbon dioxide respectively of 15, 11 and 13 volumes, average 13 volumes per 100 g. of muscle.

On resting muscle at the same partial pressure (745 mm.) a large number of experiments were performed owing to the importance of this figure for the calculation of the heat by carbon dioxide as discussed above. In Table IV only some of them are given, *i.e.* those which have

TABLE IV. Data for construction of CO_2 dissociation curve of muscle.

CO_2 partial pressure (mm. Hg)	CO_2 combined c.c. per 100 g. of muscle	CO_2 partial pressure (mm. Hg)	CO_2 combined c.c. per 100 g. of muscle	CO_2 partial pressure (mm. Hg)	CO_2 combined c.c. per 100 g. of muscle
0	2·7	134	29	500	33
23	10·3	144	26·5	520	43
34	14	150	32	745	51
36	16·5	170	32	745	44
38	17	180	10	745	53·2
45	9	180	29	745	48·7
61	20	181	41	745	47·2
65	20	300	36	745	40·2
106	24	300	38	745	49·2
110	26	320	33	—	—

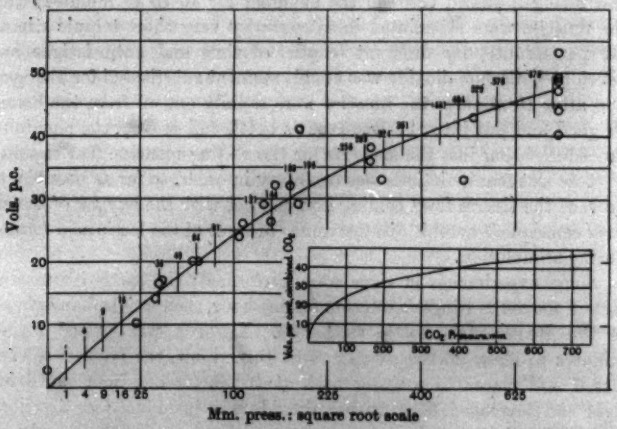

Fig. 3. CO_2 dissociation curve of muscle, from the data of Table IV: plotted on a square root scale of CO_2 partial pressure, to avoid steepness and congestion near the origin. Inset, the same plotted in the ordinary way. Muscles 45 to 50 minutes without oxygen. For fresh muscles multiply ordinates by 1·08.

been employed in Fig. 3 for plotting the dissociation curve. The temperatures of the water bath varied in the different experiments from 17° to 19° C., and an average of 18° has been assumed throughout the calculation.

The CO_2 dissociation curve, plotted in the ordinary way as in the inset of Fig. 3, rises steeply at the beginning, then gradually less steeply, finally becoming nearly horizontal. As already stated by Fenn, the curve runs much lower than that for blood; thus at a partial pressure of 50 mm. Hg of CO_2 it reaches only 18 c.c. per 100 g. of muscle, whereas in the blood of the frog it is as high as 65 to 70 volumes per 100 c.c. (see Wastl and Seliskar(14)). At 20 mm. Hg, which according to Fenn represents the partial pressure of carbon dioxide in frog's blood under normal conditions, an increase of pressure of 1 mm. will increase the amount of combined carbon dioxide by about 0·8 c.c. per 100 c.c. in the case of blood, and by only about 0·3 c.c. per 100 g. in the case of muscle. This figure confirms Fenn's results, and is in keeping with the experiments on cats by Shaw(15); it contrasts, however, with the findings of Brocklehurst and Y. Henderson(16), who attribute to human muscle a power to combine with carbon dioxide on raising the pressure of that gas in the lungs, which is very much smaller than one-third of the power of blood. It may be added here that if muscle had in fact, as they maintain, a very low capacity to combine with carbon dioxide, its hydrogen-ion concentration would be much higher than it actually is, as is easily seen by considering the Henderson-Hasselbalch equation.

The CO_2 dissociation curve is roughly of a parabolic form, and when it is plotted in the ordinary way to include the whole range up to one atmosphere pressure, the first part of the curve is so steep that it is scarcely possible to read off values in this important region. It is easier, moreover, to interpolate between points which lie approximately on a straight line. For both reasons the data of Table IV have been plotted in Fig. 3 as follows: the volumes p.c. of combined CO_2 vertically against the square root of the partial pressure of CO_2 horizontally. Theoretically the curve must pass through the origin, and we should interpret the observed point at zero pressure on the vertical axis as being due to the fact that it is impossible actually, in the time allowed, to attain zero pressure inside the muscle: the partial pressure corresponding to this volume of combined CO_2 is apparently about 1 mm.

From the curve of Fig. 3, which can be drawn with reasonable certainty between the observed points, we can read off the volume of

combined CO_2 for any partial pressure of CO_2. From this, employing the Henderson-Hasselbalch equation,

$$pH = pK' + \log \frac{[BHCO_3]}{[H_2CO_3]},$$

and assuming that the dissolved CO_2 at 18° C. is given by the usual solubility coefficient multiplied by Fenn's factor of 0·8, we may calculate the pH of the muscle corresponding to any partial pressure of CO_2. We will assume a pK' at 18° C. of 6·198, as given for human blood plasma by Cullen, Keeler and Robinson(17) and used by Fenn. The relation between pH and CO_2 pressure is shown in Fig. 4, the latter being, as

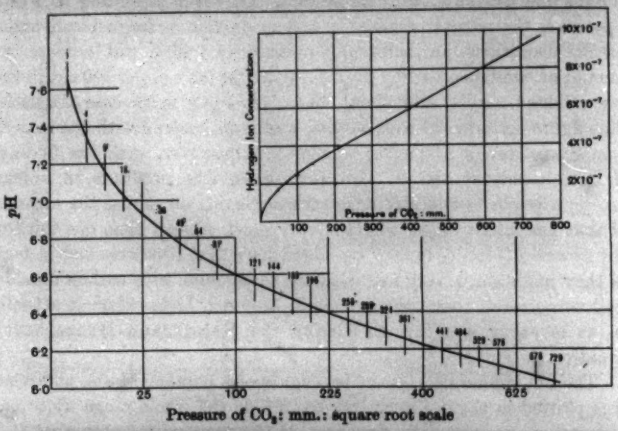

Fig. 4. The pH of muscle, calculated by the Henderson-Hasselbalch equation from the data of Fig. 3, shown as a function of the partial pressure of CO_2. CO_2 pressure on a square root scale, to avoid congestion at low pressures. Inset, the hydrogen-ion concentration (cH) as a function of CO_2 partial pressure. Muscles 45 to 50 minutes without oxygen. For fresh muscles add 0·034 to the pH, and divide the hydrogen-ion concentration by 1·08.

in Fig. 3, on a square root scale, in order to avoid undue steepness at the beginning of the curve. Inset is the relation between hydrogen-ion concentration (cH) and partial pressure of CO_2, in ordinary coordinates. We see that a muscle in an atmosphere of CO_2 has a pH of about 6, which corresponds to the lowest value which it can reach when it is removed from the body and stimulated to extreme fatigue: see Meyerhof and Lohmann(5). At a partial pressure of CO_2 of 20 mm. Hg which, according

to Fenn, represents the partial pressure of the gas in the blood of the intact living frog, the *p*H is 6·9: Fenn gives 7·2. According to Fig. 4 the CO_2 pressure required to give a *p*H of 7·0 is 18·5 mm. For a given CO_2 pressure the lower CO_2 combining capacity of muscle implies that it is appreciably more acid than blood.

The relation between *c*H and CO_2 pressure, as shown in the inset of Fig. 4, is almost exactly linear over the chief part of the range: at low pressures, however, as in blood, it bends down (see Barcroft, Bock, Hill, Parsons, Parsons and Shoji(18)). Moreover, as in blood, it can be shown that a linear relation exists between the volume of CO_2 taken up and the hydrogen-ion concentration. It is obvious, indeed, that with a CO_2 dissociation curve so similar in shape to that of blood, similar relations must, in general, exist.

It should be noted that these calculated *p*H's, and indeed all the data of Figs. 3 and 4, refer to the case of muscles kept without oxygen for 45 to 50 minutes, in which therefore lactic acid has accumulated to a small degree as described above. The true *p*H's of resting muscles are somewhat higher than those given here. If we assume that the total CO_2 capacity has been diminished by 4 volumes p.c. as the result of this lactic acid formation, and that this total capacity (see inset of Fig. 3) is about 50 volumes p.c., we may calculate roughly that in the fresh resting muscle the volume of combined CO_2 is 1·08 times as great as here given at every partial pressure of CO_2. The ordinates, therefore, of the curves of Fig. 3 should be multiplied by 1·08 throughout to represent the case of fresh resting muscle, and the hydrogen-ion concentrations calculated from them divided by 1·08. Thus in Fig. 4 the *p*H's given should be increased by log 1·08 = 0·034, if we wish to allow for lactic acid formation during the interval required for equilibration. The effect is not large, but just appreciable.

Summary.

The part played by buffers in the liberation of the heat which accompanies the new formation of lactic acid in muscle has been reinvestigated by a direct method, *i.e.* by determining the heat of combination of H_2CO_3 with living frog's muscle.

It has been found that in resting as in fatigued muscle the neutralization of 1 g. molecule of H_2CO_3 gives rise to 9400 calories. On the assumption that the heat of dissociation of muscle proteins is − 12,600 calories, as given by Meyerhof, the above value indicates that 3/4 of the neutralization is effected by muscle-proteins, and only 1/4 by other

buffers such as phosphates. 105 calories, therefore, of the 385 calories (according to Meyerhof) which accompany the formation in muscle of 1 g. of lactic acid, are to be accounted for by the process of neutralization of the acid itself. This figure is somewhat higher than that arrived at by an indirect method of Meyerhof and Suranyi, *i.e.* 80 calories per g. of lactic acid.

The CO_2 dissociation curve of a frog's sartorius, kept in an atmosphere of nitrogen and CO_2, at partial pressures of the latter ranging from 0 to 745 mm. Hg, has been constructed. The curve as a whole is about one-third as high as that given for frog's blood by Wastl and Seliskar, which implies that the inside of the muscle is appreciably more acid than blood at the same partial pressure of CO_2. At 20 mm., which is stated by Fenn to be the partial pressure in the living frog, the *p*H of muscles is about 6·9.

The relation between *c*H and pCO_2 has been plotted and found to be a linear one over the major part of a wide range of *c*H, extending from 1×10^{-7} (*i.e.* about the *c*H of a resting frog's muscle in the normal living body) to $9{\cdot}7 \times 10^{-7}$, the *c*H of muscle completely fatigued.

I am very much indebted to Professor A. V. Hill for his continual help and advice throughout all the work, and my best thanks are due to Mr J. L. Parkinson for his assistance with the heat measurements.

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INFLUENCE ON INTESTINAL MOVEMENTS OF ELECTROLYTES IN THE LUMEN OF ISOLATED SEGMENTS.

By H. E. MAGEE and B. A. SOUTHGATE.

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There is abundant evidence that the rhythmical movements of surviving intestine are markedly affected by variations in the essential constituents of nutrient fluids in contact with the serous coat; but no systematic study has apparently been made to determine whether the contractility of intestinal segments is affected by the presence or absence of electrolytes in the lumen. In the method commonly used to study the effect of substances in solution on the contractility of surviving intestine, that of Magnus, the segment is always empty except for the mucus secreted into it. This fact would seem to suggest that, for the segmenting movements at any rate, the presence of a nutrient fluid in the lumen is not essential; but it does not prove that they would not be influenced by electrolytes acting on the mucosa nor that other types of movement could not be elicited and affected by the presence of fluids of varying composition in the lumen.

Method. For this study it was essential to have a method permitting of rapid change of some or all of the lumen contents and of additions of solutions of salts to the lumen without interfering mechanically to any great extent with the segment. At the same time, it was necessary to have a continuous record of the volume changes of the segment. These requirements could not entirely be fulfilled by the various types of apparatus employed for work of a similar nature by others, *e.g.* Tyrode(1), Trendelenburg(2), Gayda(3), Baur(4), Polansky(5) and others.

A preliminary account of the method employed has been published(6), but our final procedure differs in several respects from the description given and the apparatus has been slightly modified (Fig. 1). The chief modification is the addition of an arrangement by which the nutrient fluid in the glass bath *I* is constantly renewed from the reservoir *B*, the level remaining unchanged because of the constant level arrangement attached to the outflow tube *G*. This precaution was found to be neces-

sary because the *p*H of the solution often fell very rapidly. The rate of flow found necessary to keep the *p*H steady was so slow that there was no need to warm the fluid before entering the bath. The capacity of the vessel *I* was about 150 c.c., and two such were set up in each Burn-Dale bath. Two of the latter were in use at the same time so that simultaneous records were obtained of four segments. The diameter of tube *D* should be at least 6 mm. With narrower tubes the addition and removal of fluid is much more difficult and air locks are liable to form.

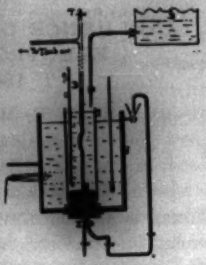


Fig. 1.

In practice the anal end of a segment, about 8 cm. long, is tied on to the lower end of *D* and then lowered into the empty bath *I* so that the segment hangs out of the opening from which the cork was removed beforehand. The oral end of the segment is then tied on to the upper end of *E* and the cork (with the tubes fixed in it) and tube *D* raised simultaneously. The cork is inserted and *D* clamped so that the segment is a little slack. Bath *I* is then filled with nutrient fluid.

After about 15 minutes rhythmical movements generally begin and only then is fluid added to the lumen. If added earlier, the segment very often remains quiescent or contracts very feebly. When filled to the desired level the side-arm of *D* is connected to the tambour, the rubber tubing *T* fixed on and the clip closed. The air column in the tambour system is kept at atmospheric pressure after withdrawal or addition of fluid. This necessitates the closing of the clip at *T* only after the rubber tubing has been in position. The lumen contents can be run off by opening the clips at *T* and on *E* and, if necessary, blowing gently down the tube. The inertia of the recording systems and the magnification of the records were kept constant as far as possible. The speed of the drum was also constant. Time marking is in minutes.

The nutrient fluids used were: for the rabbit gut, Tyrode's original fluid, for gut from the cavy, cat and ferret the solution recommended for the cavy's intestine (7). Most of the work was done on the small intestine of rabbits and cavies; but, in some of the experiments on the effect of pressure, segments were obtained from cats and ferrets.

Food was withheld for 15 hours before the animals were killed. The entire small intestine was excised and washed out with tap water.

Preliminary work with the apparatus showed that the pressure of

the lumen contents was a factor of great importance in determining the nature of the contractions. Before proceeding to study the effect of electrolytes it was, therefore, essential to investigate and, if necessary, to correct for this factor. Tyrode's fluid was placed in the lumen in the early experiments. Then it was found that physiological saline and, later, that distilled water were just as suitable. These findings at the outset suggested that the movements were not influenced by NaCl in physiological concentration, nor by the electrolytes present (in balanced concentrations) in Tyrode's solution, nor by the osmotic pressure of the lumen contents; but they did not throw any light on the effects of ions acting alone, nor on the concentration limits within which they affected the movements. For these reasons it was considered desirable to study the actions of ions in greater detail.

Effect of pressure. A measured volume of distilled water, usually 2 c.c., was placed in the lumen of each segment at first, and subsequently additions of 0·5 c.c. at a time were made. The height of the water column in *D* above the top of the fluid in *I* was measured after each addition when the segment was in diastole. This distance represented the pressure of the fluid in the segment. The gut wall gave way before the pressure to an extent depending on the capacity of the segment and probably also on its state of tone. A given pressure was always reached by the addition of a smaller volume of water in duodenal segments than in ileal segments of equal length from the same animal. The segmenting movements, present before the water was run in, continued but did not produce any movement in the water column. It was only when a certain (threshold) pressure was reached that peristaltic movements began and changes in volume became visible. Contractions began suddenly, as a rule, in segments from rabbits and cavies (Fig. 2), but more gradually in segments from ferrets and cats. As the following figures show, the threshold pressure was higher in the carnivora than in the herbivora studied. These results appeared to be directly related to the density of the muscular tissue in the gut wall.

Fig. 2. Cavy, ileum. Threshold pressure.

Animal	Duodenum	Av.	Ileum	Av.
Rabbit	0·75– 3·5	1·8 cm.	0·25–2·0	0·4 cm.
Cavy	1·50– 5·0	3·5 ,,	0·5 –3·0	1·7 ,,
Ferret	4·0 –10·0	7·0 ,,	3·0 –9·5	5·5 ,,
Cat	6·0 –12·5	8·5 ,,	3·5 –9·5	6·0 ,,

When the pressure was raised above the threshold value, the amplitude of contractions at first increased, then remained constant for a time and finally fell. Large pressures abolished all movements. The range of pressure over which no significant change of amplitude took place was more variable than in the case of the threshold pressure. In the rabbit it was approximately from 2 to 7 cm. of water and in the cavy from about 3·5 to 6·5 cm. In the ferret it varied from approximately 5 to 9 cm. and in the cat from 12 to 20. These ranges of pressure may therefore be regarded as optimal. The pressure which obliterated contractions was higher in the cat and ferret than in the rabbit and cavy. Thus with cats' gut, pressures as high as 40 cm. were required to abolish contractions, while in the case of rabbits' never more than 18 cm. were necessary, and generally much less than this. No constant differences in this connection could be distinguished between the duodenum and ileum from the same animal.

No alteration in the frequency or type of contraction at different pressures was observed, only the amplitude being affected. At pressures slightly above the threshold value fatigue did not normally set in for some hours (4–8) after the first application of the pressure. During this time the amplitude of the contractions remained approximately constant.

The establishment of a zone of optimal pressure facilitated the study of the effect of electrolytes because it showed that within limits of, for the rabbit, 5 cm. and, for the cavy, 3 cm. approximately, increase in pressure had no significant effect on movements. Consequently, the practice adopted when the effect of a substance was to be studied was to add small amounts of water to the lumen until maximal excursions of the lever were obtained. Then small amounts (0·1–0·5 c.c.) of concentrated solutions, usually 10 p.c., of salts were added and washed down the tube with an equal volume of water. The pressure was measured after such additions to avoid overstepping the optimal pressure. Control preparations were also set up several times for each salt investigated. To the controls, water only or equimolecular amounts of NaCl were added. This salt, as the earlier results showed, had no influence on movements until high concentrations were reached.

H-*ion*. The *p*H was changed by adding *N*/5 HCl or NaOH. In order to allow time for mixing, the *p*H was never measured earlier than five minutes after addition. About 0·5 c.c. was removed from the column of fluid by means of a piece of narrow hard glass tubing. This was raised to the top of the tube and about three-fourths of the fluid allowed to

run back again. Thus, contamination of the sample with acid or alkali that had not been completely washed down was avoided. The *p*H was obtained by means of the B.D.H. "capillator." The measurement was taken again after a few minutes as a check and afterwards at frequent intervals.

With water only in the lumen the *p*H remained steady for hours at its initial value of 6·4. When alkali or acid had been added it fell or rose at a rate which varied very considerably in different animals and even in different segments from the same animal. In about 50 p.c. of the animals the change was more rapid in the ileum than in the duodenum. Subsequent unpublished experiments by Macleod and one of us (H. E. M.) suggest that this was partly due to diffusion, but, perhaps, to a greater extent to neutralization by mucus.

Movements were unaffected by variations in *p*H as great as from 1·5 on the acid side to above 9·6 on the alkaline side (Fig. 3). In some cases

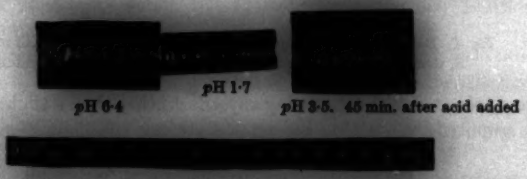


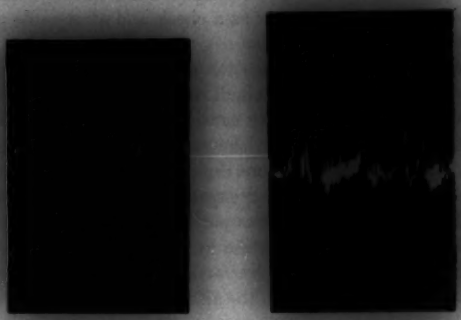
Fig. 3. Rabbit, intestine. Left and centre: absence of effect of acid 10 min. after addition. Right: another segment from same animal, no delayed effect of acid.

there occurred after several minutes a slight but transient diminution of amplitude and/or an alteration in the type of contraction which was inconstant. In others a slight transient increase in amplitude occurred soon after the change of *p*H was made.

Sodium. In freshly excised segments concentrations of NaCl up to about 3 p.c. had no immediate or delayed effect on movements (Fig. 4). Higher concentrations depressed them. In segments kept in Tyrode for some hours depression occurred at considerably lower concentrations. Duodenal and ileal segments behaved similarly.

Potassium. Concentrations of KCl below 0·5 p.c. had no effect on movements. Every further increase caused an immediate reduction in amplitude (Fig. 4). Movements were abolished with concentrations of about 2 p.c. KCl; but, if left for about 30 minutes, spontaneous recovery occurred. There was no difference between the behaviour of the duodenum and ileum.

Calcium. (Fig. 5.) The influence of $CaCl_2$ in the rabbit differed somewhat from that in the cavy. In rabbit gut concentrations up to 3 p.c.



A *B*

Fig. 4. Rabbit, ileum. *A*: top, 0·5 p.c. NaCl; bottom, 0·5 p.c. KCl in lumen. *B*, same segments: top, 2·6 p.c. NaCl; bottom, 2·6 p.c. KCl in lumen.

were tolerated without causing any effect other than a slight depression in amplitude.

Fig. 5. Cavy, intestine. Absence of effect of 0·5 p.c. $CaCl_2$ in lumen.

In the cavy concentrations of 0·9 p.c. $CaCl_2$ usually depressed contractility to a marked degree. The depression generally occurred suddenly.

There was no distinction between ileum and duodenum in either animal.

Magnesium. The cavy intestine was more sensitive than rabbit intestine to $MgCl_2$.

In four of the six rabbits on which this salt was tested no effect was

produced until high concentrations (1 p.c.) were reached, and then contractility was immediately and rapidly depressed (Fig. 6). In the

Fig. 6.

Fig. 7.

Fig. 6. Rabbit, ileum. Absence of effect of 0·2 p.c. $MgCl_2$ in lumen.
Fig. 7. Cavy, ileum. Immediately depressing effect of 0·7 p.c. $MgCl_2$.

other two a depressant action was apparent from the first addition (0·1 p.c. approx.).

In the case of the cavy no such differences in sensitivity were distinguishable, the movements being depressed markedly after the first addition (Fig. 7). The average concentration at which contractions were completely abolished was 1·4 p.c. for the rabbit gut and 0·5 p.c. for the cavy.

In both animals the ileum was slightly more sensitive than the duodenum.

Phosphates. The solution used was 10 p.c. NaH_2PO_4, the *p*H of which was adjusted to 7 by addition of NaOH.

Similar results were obtained in both rabbit and cavy.

Phosphate additions had no effect until high concentrations were reached when the large peristaltic movements gave way to very fine rapid contractions. These disappeared as the concentration was increased. The concentration which abolished all movement varied considerably. In some, 2 p.c. sufficed, but in several cases movements continued until about 6 p.c. NaH_2PO_4 was reached. If a segment thus rendered quiescent was allowed to stand, recovery of contractions occurred after about 30 minutes. The action of phosphates on both duodenum and ileum was, on the whole, the same.

Rôle of the mucous membrane. The foregoing results showed that the isolated intestine is much more resistant to the effects of unphysiological concentrations of electrolytes on the mucous side than on the serous side, and raised the question of the effects of similar concentrations of

salts after devitalization of the mucous membrane. Experience convinced us that chemical agents were more suitable for this purpose than physical measures.

Accordingly, preparations were allowed to record optimal movements for a time, the lumen contents were then drained out and replaced by the devitalizing solution. After acting for a time this was thoroughly washed out and the segment allowed to remain empty for about 30 minutes, a precaution found necessary to ensure resumption of movements after such mechanical disturbance, even in the absence of a toxic agent. Then water or 0·9 p.c. NaCl was run in until the hydrostatic pressure was the same as before. Contractions were again recorded and the solution in question added to the lumen. In all subsequent experiments HCl was exclusively employed because of the very striking effects of acid on intestinal muscle, and because the degree of acidity could be known with greater accuracy than the concentration of any salt solution.

As devitalizing agents, solutions of NaF (10–2·5 p.c.) were chiefly used, but also chloroform and alcohol vapour. The vapour was passed continuously through the lumen of the segment for a definite time by means of an air current.

In most of the experiments control segments were set up. In these the procedure was the same as in the others except that no devitalizing agent was added. In other experiments the effect of acid was determined before and after devitalization of the mucosa on the same preparation.

Results. In several experiments movements failed to restart after treatment with the devitalizing agent. In many there was complete recovery, but additions of acid failed to evoke any response. The most obvious interpretation of these two types of result was that, in the former case not only was the mucosa devitalized but also the muscular tissue—in the latter the agent had had no effect. Such findings were to be expected from the anatomical nature of the tissues. Several results were obtained of a more successful nature. These showed good recovery after withdrawal of the agent and an immediately depressing action after adding acid to the lumen (Fig. 8). No constancy could be found either in the concentration of the solution or in the time necessary to just kill the mucosa without injuring the underlying tissues. The obtaining of a successful result appeared to be merely a question of chance.

The response to NaF solutions was very striking. Immediately after addition, violent peristaltic movements set in and, on withdrawing the fluid, varying degrees of spasm over varying portions of the segment.

Spasm, when very violent, generally continued, so that it was impossible afterwards to refill the lumen.

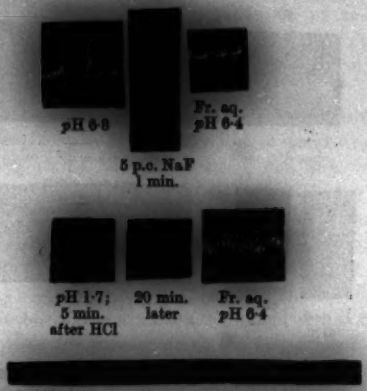


Fig. 8. Rabbit, intestine. Shows immediate effect of NaF, recovery after adding fresh water to lumen, depressing action of acid and recovery on substituting fresh water again.

Use was made of another device to test the defensive powers of the mucous membrane: If the outer nutrient fluid is replaced by Ca-free Tyrode, movements cease immediately and begin at once on adding Ca; but when $CaCl_2$ is added to the lumen instead, contractions do not begin for from five to twenty minutes, recovery being very gradual. The time varies from animal to animal and from segment to segment. It was found that when the mucous membrane had been previously killed by NaF recovery was not delayed but almost immediate (Fig. 9). Several results of this type were obtained but more were unsuccessful in the sense described above. Generally, when contractions recommenced in Ca-free outer fluid after the addition of Ca to the lumen, they were of a type different from the original: the peristaltic movements being more extensive and separated from each other by small movements (Fig. 9).

In these last experiments the circulation of the outer fluid was discontinued and measured volumes used.

Discussion. Our results on the effect of pressure confirm those of other observers, *e.g.* Baur(4), Crane and Henderson(8), Sollman and Rademaekers(9), Lurje(10) and others. The values for threshold pressure obtained by these workers and by us are not identical in all

cases, due very probably to the various types of recording apparatus used. The higher values for the duodenum than for the ileum in all

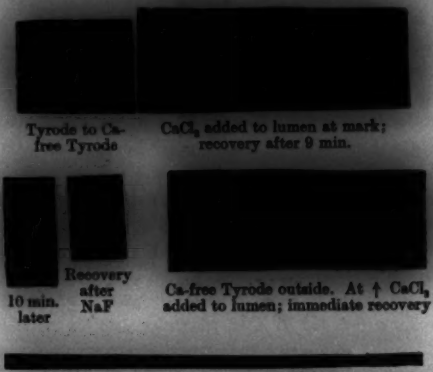

Tyrode to Ca-free Tyrode
$CaCl_2$ added to lumen at mark; recovery after 9 min.
10 min. later
Recovery after NaF
Ca-free Tyrode outside. At ↑ $CaCl_2$ added to lumen; immediate recovery

Fig. 9. Rabbit, intestine. Shows (*a*) effect of change to Ca-free outer fluid, (*b*) effect of addition of $CaCl_2$ to lumen and change in type of movements, (*c*) recovery after devitalization of mucosa by NaF (water in lumen), (*d*) change to Ca-free outer fluid and immediate recovery after adding $CaCl_2$ to lumen.

animals and for the carnivora than for the herbivora are presumably related to the density of the gut wall.

The relative indifference of the contractile processes of segments to the absence, or presence in high concentrations, of electrolytes in the lumen stands out in marked contrast with the results of similar variations in the outer environment. These findings confirm some of the experiments of Adam(11), Lurje(10), and Gayda(3), in regard to the H-ion. Further, effects when produced from the lumen were always depressant irrespective of the salt and were never specific, unless, as the experiments on Ca showed, the ion in question was absent from the outer fluid.

The results demonstrated that the tissues between the muscular coats and the lumen exert a very efficient protection against noxious substances in the cavity of the gut. There is evidence that this is so in the living animal. Thus, Wagensteen(12) has shown that extracts of the intestinal contents of normal dogs are just as toxic on injection into normal dogs as extracts of the contents of the intestines of dogs suffering from experimental intestinal obstruction and its accompanying toxæmia.

The inference that may be drawn from these experiments is that there is no abnormal production of toxins by the obstructed bowel but that the toxæmia is the direct result of devitalization of the gut wall. Cooper(13) quotes further evidence in support of this opinion, and Cruickshank(14) has also stressed the important protective function of the intestinal mucosa.

In isolated segments the chief protective rôle is most probably played by the epithelial cells owing to the absence of the circulating blood and lymph. In the living animal substances which find their way through the epithelium are presumably taken up and carried away rapidly by the circulating blood or lymph so that the submucous tissues would appear to function in a relatively passive manner. In the isolated segment it is not known precisely by what route substances in solution find their way to the muscular coats and the outer nutrient fluid. Direct passage through the submucosa and muscular and serous coats would appear more likely than the passage along the larger blood and lymph vessels and out *via* the cut mesenteric border. On this reasoning the circular muscular coat would be the first to be affected by diffusion outwards of toxic substances; but, if the rate were slow, the deleterious effects might be counteracted by diffusion inwards of outer fluid, which would serve to minimize these effects by dilution or, in the case of acids and alkalies, neutralization. The presence or absence of effects on the movements would then seem to be a question of the relative rates of diffusion of materials on the two sides of the gut wall. That the absence of effects of ions, as found by us, was due to the retarding effect of the mucous membrane on their outward diffusion is shown by the rapid and specific effects obtained by acids in the lumen after devitalization of the epithelium.

With regard to the manner in which the epithelium exerts its protective function, little of a precise nature is known. It is probable, however, that this is due in some part to secretion of mucus, a phenomena which L. Hill(15) has shown to be of great importance in the case of the respiratory passages.

The absence of immediate effects of ions in the lumen with intact epithelium rules out participation of the local nervous system in the protective mechanism. In so far as we are aware, the nerve fibres, while they ramify all through the body of the villus, have not anatomical continuity with the protoplasm of the epithelial cells; so that until these are destroyed any protective function on the part of the nervous plexus would not be called upon. The effects of fluoride in the lumen support

this opinion. These experiments showed immediate and marked stimulation of peristalsis followed by varying degrees of spasm, so that the, presumably, rapid destruction of the epithelial layer permitted equally rapid stimulation of the nerve endings. The first line of defence of the intestine, therefore, seems to be the epithelial cells and the second the neuromuscular elements which manifest this function by violent peristalsis and then spasm; both supplementing or being supplemented by the circulating fluids in the living animal.

The relatively low resistance of the gut of the cavy to Mg as found in these experiments appears to be in accordance with its great sensitiveness to this ion as demonstrated by the Magnus method (7).

Summary.

A method is described for studying the influence on intestinal movements of substances placed in the lumen of isolated segments.

The internal pressure is the factor of greatest importance in determining the response to the lumen contents. There is a threshold pressure which is higher for the duodenum than for the ileum and higher for the carnivora than for the herbivora studied.

There is an optimal zone of pressure of several centimetres of water below and above which the amplitude of contractions is less.

The contractility of isolated segments of the rabbit and cavy is indifferent to the absence from, or presence in, the lumen in relatively high concentrations of electrolytes of physiological importance; effects when they appeared were always inhibitory and not specific unless the ion in question was absent from the outer environment.

Previous devitalization of the mucous membrane led to the elicitation of immediate effects of substances placed in the lumen.

K was more depressant than Na. The cavy intestine was more sensitive to Ca and especially to Mg than the rabbit's.

The results are discussed and the opinion is expressed that the mucous membrane of the intestine exercises a very efficient protective action against harmful substances in the lumen.

We desire to express our indebtedness to Prof. Macleod and Dr Orr for their advice and criticism.

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TISSUE OXYGEN TENSION AND CARBON MONOXIDE POISONING.

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In previous papers (1, 2, 3) the effects of breathing oxygen at low pressures, of excessive bleeding and of hæmolysis upon tissue oxygen tensions were described. Using some of the same animals employed in these former researches, the effects of carbon monoxide poisoning upon tissue oxygen tensions have been investigated and the results are recorded in the present paper.

Experiments of a few hours' duration.

In some preliminary experiments the rabbit was tied down on its back for about four hours, pressure points being padded with cotton wool to render the animal more comfortable. No anæsthetic was required; with its eyes covered it kept very quiet and often appeared to sleep. Its snout was within a 100 litre gas bag containing air or other mixture of gases under investigation. The animal rebreathed the gas in the bag and the carbon dioxide expired was absorbed by soda-lime through which the gas from the bag was circulated by means of a pump. At the end of every hour about 2 litres of oxygen were added to the gas in the bag to replace that used up by the animal, which weighed about 2–3 kilos.

The oxygen and carbon dioxide tensions in the tissues were estimated by the method (4) of injection of about 200–300 c.c. of nitrogen into the abdominal cavity and under the skin, the animals being well accustomed to the injections before the commencement of the experiments. Depots of gas were thus established within the animal's body, and only one injection a week was necessary to maintain such depots. Samples of gas were withdrawn when required for analysis and the oxygen and carbon dioxide tensions in the tissues thus determined.

Control experiments were carried out in which the animal breathed only air from within the bag. It was found that tying the animal down on its back for four hours definitely changed the gas tensions under the skin, the oxygen tension being reduced always, whilst the carbon dioxide

tension was usually increased (Table I); at the same time the gas tensions in the abdominal cavity were unchanged. Evidently the soft tissues of

TABLE I. Rabbit No. 26.

Condition	Inspired gas	HbO_2 p.c. of normal	CO_2 tensions in tissues mm. Hg		O_2 tensions in tissues mm. Hg	
			Skin	Abd. cav.	Skin	Abd. cav.
Normal, not tied down	Air	100	51	51	20	37
4 hours, tied down	Air	100	62	51	13	37
4 hours, tied down	55 p.c. O_2	100	60	51	12	40
4 hours, tied down	·150 p.c. CO in air	47	50	35	1	19
4 hours, tied down	·150 p.c. CO in 55 p.c. O_2	74	64	52	9	34

the animal's back were compressed against the table so that the circulation of the blood in the tissues under the skin was interfered with. The presence of the gas under the skin did not appear to be a source of obstruction to the circulation because the pressure in the gas under the skin was only one or two centimetres of water as measured by a manometer and a similar mechanical pressure existed in the gas in the abdominal cavity. In five such experiments with different rabbits the average fall in oxygen tension under the skin was 21 p.c., whilst the oxygen tension in the abdominal cavity always remained unaltered, and thus the latter formed a very good base line for further experiments.

Three other sets of experiments were performed with the same five animals tied down on their backs. In the first set they breathed 55 p.c. oxygen, in the second set about ·150 p.c. carbon monoxide in air, and in the third set about ·150 p.c. carbon monoxide in 55 p.c. oxygen. The carbon monoxide was prepared in the usual way from formic and sulphuric acids. Details of the experiments with rabbit No. 26 are given in Table I, whilst the average results for all five rabbits are compared in Table II. In these tables the HbO_2 p.c. of normal refers to the

TABLE II. Comparison of average results for five rabbits.

Exp.	HbO_2 p.c. of normal	Tissue tensions p.c. of normal			
		CO_2		O_2	
		Skin	Abd. cav.	Skin	Abd. cav.
55 p.c. O_2	100	112	108	101	111
CO + air	42	107	78	52	56
CO + 55 p.c. O_2	62	124	115	69	83

amount of hæmoglobin not saturated with carbon monoxide. The blood saturation was estimated in initial experiments by Haldane's carmine method, but Hartridge's reversion spectroscope has been used alone

in practically all experiments described in this paper; the curve employed for the spectroscope was built up from the blood of rabbit No. 35 which has been used exclusively for such standard purposes throughout the present research.

When breathing 55 p.c. oxygen both the carbon dioxide and oxygen tensions in the tissues were usually increased (Table II). In this table 21 p.c. has been added to the figures obtained for oxygen tension under the skin for the reason given above, but as there was no means of judging whether this correction was sufficient, attention is directed chiefly to the figures for the tensions in the abdominal cavity where no correction was needed. It will be seen that the oxygen tension therein was increased by 11 p.c. when breathing 55 p.c. oxygen; that it was reduced to 56 p.c. of the normal value when breathing ·150–·200 p.c. of carbon monoxide in air, whereas when a similar quantity of carbon monoxide was breathed in 55 p.c. oxygen, the oxygen tension in the abdominal cavity fell to only 83 p.c. of its normal value, indicating clearly how the breathing of oxygen at higher pressure relieved the condition of oxygen deficiency in the tissues.

These preliminary experiments were of too short a duration for comparison with my previous results for bleeding and hæmolysis when the experiments covered from four to six days.

Experiments of from four to six days' duration.

Full details of the bleeding and hæmolysis experiments with rabbits Nos. 37, 32, 22 and 23 will be found in a previous paper(3); No. 32 was a splenectomized animal. In these previous experiments a gradual lowering of the hæmoglobin content of the blood was produced by several daily bleedings from an ear vein or by injection of hæmolytic serum, so that by the fourth or sixth day the hæmoglobin was much reduced.

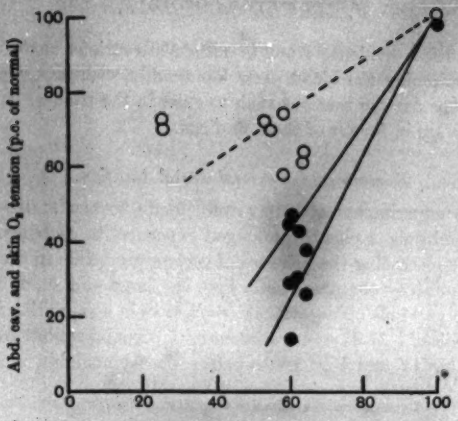
In the present experiments the same animals—but now a year older—were enclosed separately in the respiration chamber of about 150 litres capacity, which has been described elsewhere(1). They breathed carbon monoxide in gradually increasing percentage up to about ·110 p.c. of an atmosphere, for from four to six days. Sufficient food and water were enclosed in the chamber to last this period and the animal was quite free to move about inside the chamber. At the end of the fourth or sixth day the chamber was opened and immediately a drop of blood was obtained from an ear vein and placed at once in distilled water in a tube from which air was excluded. The animal was thus exposed to air for a few seconds only before the blood was withdrawn. During the next

few minutes gas samples were withdrawn from the abdominal cavity and from under the skin. Observations proved that on breathing air again, the oxygen and carbon dioxide tensions in the gas depots in the tissues changed so slowly that no material alteration in composition could be detected during the first ten minutes after opening the chamber. There were about 200–250 c.c. of gas present in the depots, and such a quantity would not permit of a very rapid change in composition in such experiments. The details of the experiments are given in Table III

TABLE III. Effects of carbon monoxide compared with those of bleeding and hæmolysis.

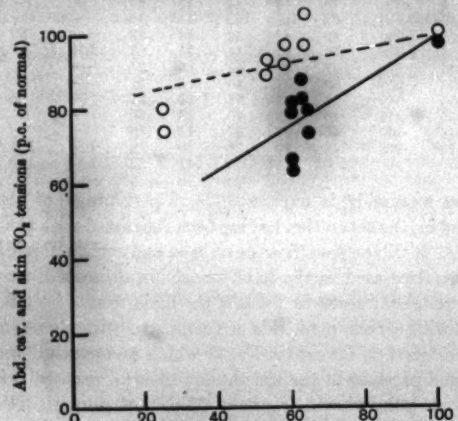
					CO_2 tensions in tissues mm. Hg				O_2 tensions in tissues mm. Hg			
			HbO_2 p.c. of normal		Skin		Abd. cav.		Skin		Abd. cav.	
Rabbit No.	Exp.	Day of exp.	Before exp.	After exp.	Before exp.	After exp.	Before exp.	After exp.	Before exp.	After exp.	Before exp.	After exp.
37	Bleeding	4th	100	25	53	43	52	39	20	14	38	28
	CO	4th	100	60	49	40	47	37	21	6	38	18
32	Hæmolysis	4th	100	63	48	51	50	49	22	14	34	21
	CO	4th	100	60	48	32	44	28	15	2	35	16
22	Hæmolysis	6th	100	53	55	51	51	50	20	15	38	22
	CO	6th	100	62	48	40	47	42	26	8	39	17
23	Hæmolysis	6th	100	58	49	44	48	45	25	18	39	28
	CO	6th	100	64	46	34	47	37	22	7	42	16
55	CO	4th	100	60	51	39	50	37	22	5	36	13
56	CO	4th	100	60	51	46	49	42	21	9	42	18

and the results are compared more clearly in Figs. 1 and 2. There was no doubt that in the first few days of carbon monoxide poisoning the oxygen tensions in the tissues were lowered to a more marked degree than they were during the first few days of bleeding and hæmolysis; the carbon dioxide tensions were also lower in the former than in the latter conditions. The rabbits were one year older when the carbon monoxide experiments were performed, but this was not responsible for the differences observed, because two very young rabbits, Nos. 55 and 56, showed similar low absolute figures for oxygen tensions in the tissues in carbon monoxide poisoning (Table III). I have already demonstrated(3) that exposure to low oxygen pressure in the air had about the same effect upon tissue oxygen tensions as did bleeding and hæmolysis, so that for a similar loss in function of hæmoglobin carbon monoxide poisoning deprived the tissues of oxygen to a greater extent than did any of the other conditions studied. J. S. Haldane(5) suggested some years ago that such an effect might underlie the fact that the symptoms exhibited in carbon monoxide poisoning were unexpectedly severe for the degree of saturation of the blood with the gas, and J. B. S. Haldane(6)



Hæmolysis and bleeding, ○. Carbon monoxide, ●.

Fig. 1. Relationships between Hb content (p.c. of normal) and oxygen tensions in the abdominal cavity and under the skin in bleeding and hæmolysis; also relationships for the same rabbits between Hb percentage unsaturated with carbon monoxide, and tissue oxygen tensions, the upper continuous line being drawn through the average tension for the abd. cav. and the lower line through that for the skin.



Hæmolysis and bleeding, ○. Carbon monoxide, ●.

Fig. 2. General arrangement as for Fig. 1, but carbon dioxide tensions in the tissues (p.c. of normal) forming the ordinates.

obtained indirect evidence from *in vitro* observations that in carbon monoxide poisoning the blood parts less readily with its oxygen when exposed to low oxygen tensions such as exist in the tissues. My *in vivo* observations are in favour of these findings.

Experiments of several weeks' duration.

In these experiments attempts were made to acclimatize rabbits, rats, mice and guinea-pigs to prolonged exposures to carbon monoxide by gradually increasing the pressure of carbon monoxide in the inspired air. The respiratory chambers used were the large and small chambers employed in previous observations (1, 2) on oxygen acclimatization.

Full details of a 37 days' experiment with a young rabbit, No. 54, are given in Table IV and Fig. 3. As before the hæmoglobin unsaturated

TABLE IV. Attempted acclimatization to carbon monoxide; rabbit No. 54.

	CO p.c. atmosphere				CO_2 tensions in tissues mm. Hg		O_2 tensions in tissues mm. Hg						
Time (days)	Inspired air	Abd. cav.	Skin	HbO_2 p.c. of normal	Skin	Abd. cav.	Skin	Abd. cav.	Weight kilos	Hb p.c.	R.B.C. mills. p.c. mm.	W.B.C. thous. p.c. mm.
0	0	0	0	100	47	46	19	32	2·16	64	5·0	17·0
3	·065	·035	·040	74	43	42	16	26	—	—	—	—
7	·080	·040	·050	70	44	44	15	23	—	—	—	—
10	·120	·050	·055	63	41	43	15	23	—	—	—	—
14	·120	·050	·050	60	42	42	14	23	—	80	7·5	18·0
17	·140	·055	·055	55	43	44	14	23	2·12	—	—	—
21	·140	·050	·055	57	40	42	14	21	—	—	—	—
24	·165	·060	·055	50	37	40	14	21	—	—	—	—
28	·160	·055	·055	52	37	37	14	21	—	95	8·5	14·0
31	·170	·055	·055	49	39	40	13	20	—	—	—	—
35	·225	·065	·060	43	37	37	13	19	—	—	—	—
37	·240	·070	·065	43	39	36	10	16	1·85	100	9·0	19·0
38	0	—	—	100	45	43	41	53	1·85	100	9·0	19·0

with carbon monoxide is expressed as a percentage of the normal oxyhæmoglobin, the saturation having been estimated with the reversion spectroscope. It will be seen that the oxygen and carbon dioxide tensions in the tissues decreased as the oxyhæmoglobin decreased, the relationships being roughly linear. In Table V the main results for this 37 days' experiment with carbon monoxide are compared with those obtained in a previous research (1) for rabbit No. 2, which was exposed for 39 days to low oxygen pressure in the air, the lowest level reached being about 7·5 p.c. of an atmosphere, or about 33 p.c. of normal. The highest saturation of the blood of rabbit No. 54 with carbon monoxide was 57 p.c., so that the HbO_2 was 43 p.c. of the normal. There is no way to determine whether this implies a lower functioning power of hæmoglobin than the

former figure of 33 p.c. in the condition specified. In both cases there was a marked decrease in oxygen and carbon dioxide tensions in the

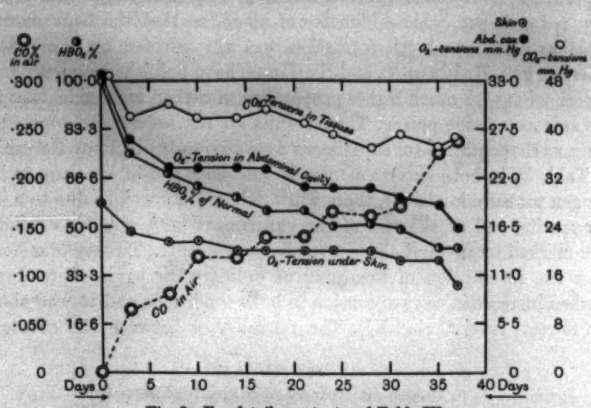


Fig. 3. For details see text and Table IV.

tissues; the carbon dioxide tensions in the tissues appeared to be more greatly depressed in the low oxygen experiment, indicating a greater

TABLE V. Comparison of effects of 39 days' exposure to low O_2 pressure (lowest = 7½ p.c. O_2) in the inspired air with those of 37 days' exposure to carbon monoxide (highest saturation of blood = 57 p.c.).

	Rabbit No. 2 under low O_2 pressure	Rabbit No. 54 under carbon monoxide
O_2 tension in abd. cav., p.c. of normal	63	50
O_2 tension under skin, p.c. of normal	54	53
CO_2 tension in abd. cav., p.c. of normal	41	78
CO_2 tension under skin, p.c. of normal	43	83
Red blood corpuscles, p.c. of normal	173	180
Hb, p.c. of normal	133	156
Weight, p.c. of original	88	85

increase in volume respired. Such an increase in breathing would perhaps not be of such value when breathing carbon monoxide in air. Individual differences in responses must also be remembered.

Nasmith and Graham(7), in similar acclimatization experiments with guinea-pigs, demonstrated that by increasing the quantity of hæmoglobin and number of erythrocytes the animals were able to compensate in some degree for reduction in oxygen carrying capacity of blood due to carbon monoxide. In my rabbits there was also a marked

increase in the hæmoglobin percentage and red cell count per cubic millimetre of blood (Table V); at the same time in the carbon monoxide experiment there was an increase of over 80 p.c. in the reticulated or new red cells per cubic millimetre of blood, so that the bone marrow function of new red cell formation was not affected by the poison. Warburg(8), Haldane(9), and Keilin(10) have published evidence from experiments with much higher proportions of carbon monoxide, that the gas acts as a tissue poison. These results are not necessarily opposed to mine, as the conditions of experiment were obviously different. Returning to Table V, in both carbon monoxide poisoning and in exposure to low oxygen pressure in the air, there was a fall in body weight due to a loss of appetite, and at the end of the experiment both animals exhibited well-marked tetany and also convulsive movements. Twenty-four hours after the resumption of breathing of ordinary air again there was a marked increase in oxygen tension in both conditions and to well above the normal level (Table VI). The resemblance between the effects of

TABLE VI.

		O_2 tensions in tissues mm. Hg			
		Skin		Abd. cav.	
Rabbit No.	Exp.	Before exp.	First day after cessation of exp.	Before exp.	First day after cessation of exp.
2	Low O_2	26	40	44	55
54	CO	19	41	32	53

exposure to low oxygen pressure in the air and of carbon monoxide poisoning was therefore striking.

The carbon monoxide in the inspired air was estimated by a modification of one or other of the bottle or flask methods as used by Haldane(11), Hartridge and Barcroft. The air containing the carbon monoxide was forced, under mercury pressure, into a small inverted flask—135 c.c. capacity—filled with and held over mercury. The neck of the flask measured 5·5 cm. in length and 1·5 cm. in internal diameter. After filling the flask with the gas a rubber stopper was inserted and 5 c.c. of diluted blood injected from a syringe by means of an hypodermic needle forced through the stopper. Blood always from the same rabbit, No. 35, was used to make up the dilute solution. The hæmoglobin value of the blood of this rabbit was only about 60–65 p.c. of the usual scale, so that instead of making up the 5 c.c. blood solution to 1 in 200 it was about 1 in 130, ammonia being added in the usual quantity. The flask, protected from the light, was then shaken vigorously by hand for

15 minutes including the usual short intervals for rest. The temperature of the gas was about 17° C. and the barometric pressure about 755 mm. Hg. The percentage saturation of the blood with carbon monoxide was estimated with the reversion spectroscope and the percentage present in the air read off from the curve, Fig. 4, built up from equilibration

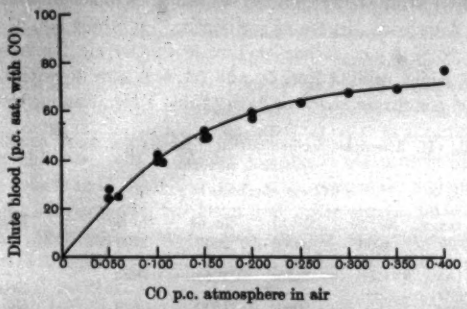


Fig. 4. Relationship between carbon monoxide percentage in air and percentage saturation of blood of rabbit No. 35 with carbon monoxide *in vitro*.

values, using the blood of rabbit No. 35. In building up this curve equilibrations were carried out in flasks of both 500 c.c. and 135 c.c. capacity, the larger flask being used as a control, since it required about four times the quantity of carbon monoxide used for the smaller flask. The small quantities of carbon monoxide were measured over water in a water jacketed burette, made out of a 1 c.c. standard pipette, and connected up with a water leveller. After adjusting this leveller the small quantity of carbon monoxide required was drawn through a three-way glass tap by a small glass syringe containing a small quantity of distilled water, and the actual amount withdrawn was read off from the burette. The gas in the syringe was never opened to the air, as the syringe formed the third and single attachment to the three-way glass tap, which was similar to that at the end of an ordinary gas sample tube. The other two and parallel attachments to this tap were the burette itself and an hypodermic needle filled with distilled water by means of which the carbon monoxide was transferred from the syringe to the flask by way of the rubber stopper; this flask contained a small quantity of distilled water through which the carbon monoxide was seen to bubble. By means of the water and the syringe the passages were well flushed out several times. The results from the two flasks agreed very closely, indicating

that the measurements of the small quantities of carbon monoxide were reasonably accurate. A few controls were also carried out by means of a mixture of gas made up in an 100 litre gas bag. I can find in the literature only a very few such results for rabbits' blood, namely those published by Haldane[12] and Krogh[13].

Some interesting facts regarding the influence of acclimatization were obtained. After some degree of acclimatization rabbit No. 54 tolerated from ·225 to ·240 p.c. carbon monoxide in the air for several days (Table IV), whilst rabbits Nos. 52 and 53 were able to withstand from ·250 to ·300 p.c. for at least six days (Table VII). Similar results were

TABLE VII. Attempted acclimatization of rabbits Nos. 50, 51, 52 and 53 to carbon monoxide.

Time (days)	CO in inspired air p.c. atmos.	Weight of rabbits in g.				Deaths
		No. 50	No. 51	No. 52	No. 53	
0	·100	2000	2300	2000	2700	—
7	·125	—	—	—	—	—
15	·130	1780	2430	1950	2820	—
23	·200	—	—	—	—	No. 50
30	·200	—	2000	1900	2820	No. 51
43	·200	—	—	1860	2650	—
46	·250	—	—	1900	2650	—
49	·300	—	—	—	—	—
51	·300	—	—	1800	2850*	Nos. 52 and 53 killed for histology

* The apparent increase in weight in this animal was due to fluid in the pleural and abdominal cavities.

TABLE VIII. Attempted acclimatization of six guinea-pigs, six rats and twelve mice to carbon monoxide.

Time (days)	CO in inspired air p.c. atmos.	Average weight in g.			Deaths
		Guinea-pigs	Rats	Mice	
0	·070	560	76	21·5	—
15	·110	—	—	—	1 guinea-pig
18	·110	492	71	18·6	2 rats
19	·130	—	—	—	—
22	·200	—	—	—	—
25	·200	—	—	—	1 rat
27	·250	—	—	—	1 guinea-pig
28	·250	—	68	—	1 mouse, 1 rat; remaining 2 rats killed for histology
32	·250	427	—	17·4	1 mouse
34	·250	—	—	—	4 guinea-pigs
36	·250	—	—	17·5	2 mice
46	·250	—	—	—	—
54	·250	—	—	16·8	3 mice

obtained with rats, mice and guinea-pigs (Table VIII). The experiments with guinea-pigs confirm and extend those described by Nasmith and Graham[7]. All these results indicated the marked influence of

attempted acclimatization, because quite unacclimatized animals were unable to tolerate ·150 p.c. of carbon monoxide for three days. It must be remembered, however, that in the so-called acclimatized animals, in spite of their increased resistance, histological evidence of disintegration of the tissues was present. Such changes have not been appreciated fully in considering acclimatization. Careful histological examination of my animals revealed changes resembling very closely those I described a short time ago(14) as effects of prolonged exposure to low oxygen pressure in the air. Full details of these pathological changes in carbon monoxide poisoning will be published elsewhere, but the main changes, stated briefly, were excessive congestion of the liver with marked atrophy of the liver cells, a similar change in the muscle fibre of the heart particularly near the epicardium, often congestion and emphysema of the lungs, congestion of the brain and other organs, fatty degeneration of the heart and liver in some rabbits and guinea-pigs, often œdema of the tissues and even dropsical fluid in the pleural, peritoneal and pericardial cavities.

Saturation of the blood in vivo *with carbon monoxide.*

In Table IX are collected together some of the results for saturation of the blood of various rabbits with carbon monoxide after breathing

TABLE IX. Saturation of blood of rabbits *in vivo* with carbon monoxide.

Rabbit No.	Date	Duration	CO in inspired air p.c. atmos.	Saturation of blood with CO p.c.
23	20. ii. 29	96 hours*	·110	36
	18. iii. 29	47 hours	·075	30
	26. iii. 29	96 hours	·200	53
37	11. ii. 29	96 hours*	·110	40
	28. ii. 29	24 hours	·050	18
	22. iii. 29	44 hours	·175	44
22	26. ii. 29	144 hours*	·125	38
	28. ii. 29	42 hours	·170	46
	20. iii. 29	44 hours	·075	28
32	15. ii. 29	96 hours*	·110	40
52	19. iii. 29	43 days*	·200	50
	27. iii. 29	51 days*	·300	60
53	19. iii. 29	43 days*	·200	52
	27. iii. 29	51 days*	·300	60
55	31. v. 29	96 hours*	·110	40
56	7. vi. 29	96 hours*	·100	40

* CO p.c. was increased gradually throughout this period to reach the final figure quoted.

various percentages of the poison for periods varying from 24 hours to 51 days. Similar figures for rabbit No. 54 are recorded in Table IV. All these results are plotted in Fig. 5 where, it will be seen, they follow fairly

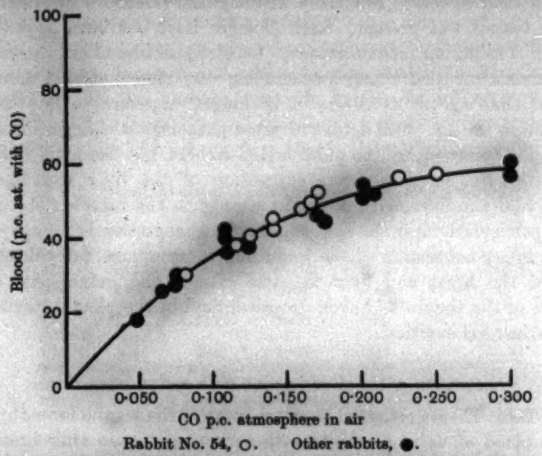


Fig. 5. Relationship between carbon monoxide percentage in the inspired air and the percentage saturation of blood of various rabbits *in vivo*.

closely a single curve. This implies that acclimatization exerted little or no influence upon the saturation of the blood with carbon monoxide, because the points obtained after one or two days' exposure followed the same curve as those obtained after forty or fifty days' exposure.

Tension of carbon monoxide in the tissues.

An attempt has been made to estimate the carbon monoxide tension in the tissues from its tension in the depots of gas within the animal. About 150 c.c. of gas were withdrawn from the abdominal cavity and from under the skin during the first few minutes after opening the respiratory chamber, and the carbon monoxide tension was estimated in this gas, which was passed over mercury into a small flask of 135 c.c. capacity such as was described above, and 5 c.c. of diluted blood were injected through the rubber stopper. The blood was then saturated with the carbon monoxide in the gas by vigorous shaking, the main outline being similar to that for the flask technique already described. The gas

from the tissues contained about 5 p.c. carbon dioxide and 1, 2 or 3 p.c. oxygen. It was found necessary to add about twice the usual quantity of ammonia to the diluted blood to maintain the necessary alkalinity to prevent coagulation during agitation, the carbon dioxide being absorbed and disappearing from the gas. The diluted blood contained about 2½ p.c. ammonia. During agitation a considerable froth was formed, but on standing sufficient fluid soon accumulated in the neck of the inverted flask and the bands were quite distinct on spectroscopic examination. After estimating the percentage saturation of the blood with carbon monoxide by means of the reversion spectroscope the tension of the carbon monoxide was read off with the aid of three curves given in Fig. 6. These curves were built up at 17° C. and 755 mm. Hg by means of the same flask technique, the gas mixture containing about 5 p.c. carbon dioxide and also 1, 2 or 3 p.c. oxygen in addition to the necessary

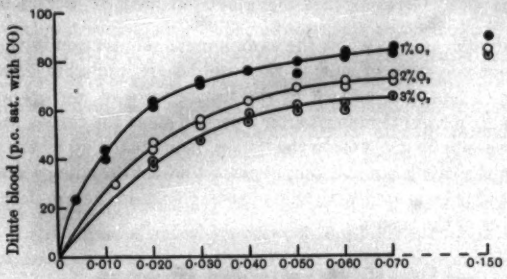


Fig. 6. Dilute blood, equilibrations at 17° C.; for details see text.

quantity of carbon monoxide measured by the burette previously mentioned. Both 500 c.c. and 135 c.c. flasks were used, the former as a control for the latter to avoid possible errors due to measuring very small quantities of carbon monoxide.

Great care was taken to prevent errors due to leakage and entrance of air, all tubes, etc. being filled carefully with mercury. In all cases at the end of a saturation and an estimation a sample of gas from the flask was analysed to prove that the composition of the gas as regards oxygen content had not changed during the manipulations. The points in Fig. 6 reveal some scatter, but this was not great and probably due to the fact that some points on each curve were determined on different days spread out over several months, blood from rabbit No. 35 being

used throughout. The method described for equilibrations with whole blood at 37° C. by Douglas, Haldane and Haldane[15] was also tested, but it was not convenient for the transference of the small quantity of gas (150 c.c.) available.

The carbon monoxide tension in the tissues as estimated by the above method is shown in Table IV for rabbit No. 54 and in Table X for some

Table X. Carbon monoxide tensions in tissues.

Rabbit No.	Duration (hours)	CO in inspired air p.c. atmos.	CO in abd. cav. gas p.c. atmos.	CO in gas from under skin p.c. atmos.
22	43	·075	·025	·035
23	48	·075	·030	·040
56	96	·100	·045	·045
55	96	·110	·040	·035
37	48	·175	·050	·050
32	48	·200	·050	·050
23	96	·200	·060	·055
37	48	·250	·050	—

other rabbits. On the whole the carbon monoxide tension in the tissues was about one-third of that in the inspired air, probably because the tensions (1–3 p.c.) of oxygen in the tissues were so much lower than that (14–21 p.c.) in the lungs; after a certain stage the carbon monoxide would cease to be given off to the tissues, being taken up by the reduced blood in the capillaries and simply carried back to the lungs again. The tensions thus estimated were those existing outside the capillary wall in the spaces of the tissues.

Value of factors of so-called acclimatization.

It has been stated above that by gradual acclimatization of an animal to carbon monoxide poisoning the animal was made to tolerate for at least several days a percentage (·250) of the poison in the air much greater than that (·150) which was usually fatal if breathed for three days without some previous acclimatization. In an unacclimatized animal the oxygen tension in the abdominal cavity fell from about 35–40 mm. Hg to about 16–20 mm. Hg when breathing from ·100 to ·150 p.c. of carbon monoxide (Table III), whereas in an acclimatized animal the same low level for tissue oxygen tension in the abdominal cavity was not attained until breathing about ·250 p.c. carbon monoxide (Table IV). In acclimatization then, although the animal was not able to prevent its tissue oxygen tension from falling, the fall was less for a given percentage saturation than in the unacclimatized animal. It has been observed that in carbon monoxide poisoning a rabbit cannot

tolerate so low an oxygen tension as 16–20 mm. Hg in the abdominal cavity for any length of time without risk of death. A similar limit was obtained for animals whilst breathing oxygen at low pressure in the air, and also with bleeding and hæmolysis.

What are the factors of value in so-called acclimatization? There was increased breathing by means of which the oxygen pressure in the alveoli of the lungs might have been increased from 14 to about 20·9 p.c.; the lower carbon dioxide tension in the tissues gave evidence of this (Tables IV and V). Then there was the great increase in red cells and hæmoglobin per cubic millimetre of blood, that is in oxygen capacity per cubic millimetre. In spite of these two great advantages—increase breathing and increased hæmoglobin—there was the marked decrease in oxygen tension in the tissues and also definite histological evidence of disintegration in the cells of the tissues. My results are not in favour of other factors of great importance in resistance to carbon monoxide due to acclimatization, apart from adjustments to normal *p*H and the ability of the vital organs, particularly the heart, to continue to function under the low oxygen tension in the tissues. All the pathological evidence, the value of which has previously been underestimated, indicated that circulatory failure was present in some degree even in animals which appeared to be more or less acclimatized. The heart muscle appears to be the chief factor determining the power to tolerate severe carbon monoxide poisoning just as in the case of very low oxygen pressure. Probably the adjustments which occur in the heart muscle in so-called acclimatization are similar to those which occur in this muscle during training of athletes; they have not yet been determined accurately. Of course all tissues must be affected by the abnormally low oxygen tension, but it is the heart muscle which plays the chief part in determining the level to which this tissue oxygen tension will fall.

Summary.

1. During the first few days of carbon monoxide poisoning the tissue oxygen tensions are lowered to a more marked degree than by a similar loss of function of hæmoglobin due to bleeding or hæmolysis; this agrees with J. S. Haldane's prediction. A similar, but not so marked, difference applies to the carbon dioxide tensions in the tissues.

2. It is possible for animals (rabbits, rats, mice, guinea-pigs) to tolerate about ·250 p.c. of carbon monoxide in the air for several days after some degree of acclimatization, whereas ·150 p.c. of the gas is not tolerated for three days by unacclimatized animals.

3. Even when an animal is exposed to gradually increasing percentage of carbon monoxide, the tissue oxygen tensions are abnormally low, although there is increased breathing and also great increase in the hæmoglobin content per cubic millimetre of blood. At the same time the tissues show marked histological evidence of disintegration, extreme congestion of the liver with atrophy of liver cells being the outstanding feature, for which heart failure is held mainly responsible. Power to tolerate carbon monoxide depends upon the powers of the vital organs, particularly the heart, to continue to function under a low tissue oxygen tension.

4. The resemblance between the effects (physiological and pathological) of carbon monoxide poisoning and of exposure to low oxygen pressure in the air is striking.

5. The carbon monoxide tension in the tissues has been measured; usually it is about one-third of that in the inspired air. The low carbon monoxide tension in the tissues is due to the very low oxygen tension (1–3 p.c.) which exists in the tissues, the reduced blood in the capillaries carrying the carbon monoxide back again to the lungs.

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THE PRESENCE OF HISTAMINE AND ACETYLCHOLINE IN THE SPLEEN OF THE OX AND THE HORSE.

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I. Introductory.

The fact that extracts from different animal organs (liver, lung, voluntary muscle) exhibit physiological activities resembling those of histamine and choline, and contain those bases in quantities sufficient to account for these effects, has been demonstrated in previous communications from this Laboratory (1, 2). When due allowance is made for the inevitable losses in the rather difficult processes of separation, the quantities isolated and chemically identified have been sufficient, indeed, to warrant a more general presumption that, when activity of either of these types can be clearly demonstrated in an extract from any fresh animal organ, it is probably due to the presence of the corresponding simple basic principle. It appeared to us desirable, however, to apply the methods, successfully used in the cases already investigated, to the extract of one additional organ, namely the spleen. Among the many publications dealing with the activities of organ extracts there are several concerning those obtained from the spleen. Oliver and Schafer (3) mention a fall followed by a gradual rise of the arterial pressure of the dog, as the result of injecting such an extract intravenously. Zuelzer (4) claimed to have extracted from the gastric mucosa, taken during digestion, a hormone which stimulated peristalsis of the intestine. The proprietary preparation "hormonal," made at his direction, appears, however, to have been made from the spleen, on the supposition that this organ acted as a depot for the hormone produced by the stomach. The makers supplied it as a spleen extract to Berlin (5). Berlin's investigations were chiefly concerned, however, with an extract of fresh spleen prepared by himself, by boiling the minced tissue with water for an hour. From this, after purification, he precipitated the bases as phosphotungstates and fractionated them by Kutscher's method. Fraction A was found to have

an activity resembling that of choline, and the latter base was isolated from it as the chloroaurate, of which 17·5 g. (corresponding to 4·78 g. of choline) were obtained from 23 kg. of spleen substance. Fraction B had an activity resembling that of histamine, and this accounted for a large part of the total activity of the extract; but, since the active principle was not precipitated at the appropriate stage of the silver fractionation, Berlin concluded that the substance was not histamine itself, and that this base was, indeed, not present in the extract. From "hormonal" he separated two similar fractions, but without isolating the base in either case. Stern and Rothlin(6), who had compared the actions of a series of organ extracts on isolated arterial strips and other forms of plain muscle, found that the extract of spleen had a specially powerful stimulant action. From a watery decoction of spleen, purified by evaporation and solution in alcohol, they obtained a preparation with a relatively high activity. The active principle was soluble in alcohol, insoluble in ether, not precipitated by tannin, but completely by phosphotungstic acid. The physiological effects of the preparation were strongly reminiscent of those produced by histamine; but, since they found that the activity was destroyed by heating with alkali, and that hot chloroform would not extract it completely from the residue obtained by evaporation with sodium carbonate, Stern and Rothlin were of opinion that the principle was not histamine itself, and provisionally named it "lienine."

In other publications, many concerned with "hormonal," the suggestion can be found that the activity of spleen extracts is due to choline, or histamine, or similar active principles.

We knew from our own experience, previously recorded(1), that a principle, occurring in a complex mixture of tissue bases, may show apparent differences in its chemical behaviour from that of pure histamine, as great as those described by Berlin and by Stern and Rothlin, and yet be histamine itself. It seemed desirable to examine anew the question of the chemical identity of the histamine-like constituent of spleen extracts. The investigation undertaken with this object has led to additional results of unexpected interest.

II. Recognition of two vaso-dilator principles.

As in our work on other organs, we have used extracts prepared by mincing the fresh spleens directly into cold alcohol, in place of the watery decoctions with which earlier investigators have started. We have mentioned the fact that Berlin isolated choline from his extracts in

relatively high proportion; and, although his prolonged boiling of the tissue with water might conceivably have contributed to this result, we were prepared for the possibility that our alcoholic extracts might also show a relatively large "choline" component in their depressor action. We found, indeed, when the depressor action of the extract was measured, as usual, in comparison with that of a standard histamine solution on the arterial pressure of a cat under ether or urethane, that a surprisingly large proportion of the original effect of the extract was eliminated by administering atropine. This activity, in terms of choline, was determined by the following method. The histamine-like effect of the extract was first determined, in terms of pure histamine, on an atropinized cat under ether. On another cat, similarly anæsthetized, but without atropine, the same dose of spleen extract was again compared, as regards depressor action, with the same dose of histamine. The effect of the spleen extract being now much stronger, choline in varying doses was added to the constant dose of histamine, until this mixture again matched the dose of spleen extract in effect. The quantity of choline thus indicated as present in the spleen extract was unexpectedly and, indeed, almost incredibly great. Drs W. Bauer and Dickinson Richards, who were working with us at the time, carried out an assay of the type described on the extract from our first large batch of 50 kg. of ox spleen, from which, in due course, histamine was isolated. Their estimate showed a "histamine" activity corresponding to 0·42 g. of histamine, or about 8 mg. per g., and, in addition, a "choline" activity corresponding to 116 g. of choline, *i.e.*, more than 2 g. of choline per kg. of fresh spleen substance. Our immediate object, at that time, was the isolation of the histamine-like constituent; but we noted that the choline action was practically unchanged by the purification with basic lead acetate, being estimated as equivalent to a total of 112 g. of choline, while 95 p.c. of it disappeared in recovery of the mixed bases from their phosphotungstates by the use of baryta. The lost activity was not in the phosphotungstic acid filtrate, and the suggestion was obvious that we were dealing, not with choline itself, but with some much more active derivative of choline, having a high sensitiveness to treatment with alkali. This suggestion was readily confirmed by treatment of samples of the original extract with alkali. One sample was made alkaline to *p*H 10, and left at room temperature for 48 hours. On re-testing after this treatment it was found to have retained its histamine action unimpaired, while the "choline" action had practically disappeared; the volume of extract matching a given dose of pure histamine in depressor

action was now not significantly changed by administering atropine, whereas before the alkali treatment it had been more than doubled. Addition to the original extract of an equal volume of normal sodium hydroxide produced a similar destruction of the "choline" component of the depressor action, in ten minutes at room temperature.

The main bulk of this large-scale extract of ox spleen had already passed through the stage of purification at which this alkali-sensitive principle was almost entirely lost. We decided, accordingly, in the further investigation of this batch, to follow the histamine-like component only. In due course, by the procedure described in detail in a later section, we isolated this, and found it, as in other organ extracts, to be histamine itself. Our original objective was thus attained; but the evidence of an alkali-sensitive principle with a choline-like action raised possibilities of new and unexpected interest, requiring different methods of attack.

We satisfied ourselves, in the first place, that choline itself was not present, in our first large-scale extract, in quantities of the order required to produce the observed effects. The most obvious possibility was that we were dealing with an unstable ester of choline, having an activity so much greater, that hydrolysis would practically annul it. Among the esters of choline which had been examined, only acetylcholine was known to have the required characteristics of intense activity and extreme liability to hydrolysis in alkaline solution. If the principle were, indeed, an ester so unstable as this, the fact that its presence had not been detected by the earlier observers, who made their spleen extracts by prolonged boiling with water, needed no explanation. It seemed unlikely that our own extraction with alcohol, made without reference to the presence of so unstable a substance, would have preserved it all without decomposition. We accordingly made a series of experiments, in which we had valuable help from Mr H. P. Marks, in order to determine what additional precautions were desirable. These experiments were greatly facilitated by the opportunity of obtaining spleens, immediately after death, from horses killed at the Farm Laboratories of the National Institute, through the valuable co-operation of our colleague Major G. W. Dunkin. In a few experiments we froze the spleens immediately on removal, by immersing them in alcohol cooled to about — 60° C., and minced them while still frozen, returning the mince immediately to the cold alcohol, which rose slowly to the ordinary temperature as the extraction proceeded. We obtained good yields of the choline-derivative in this way, but not better than in other trials in

which such precautions were omitted. The one essential condition for a good yield was found to be the *immediate* mixing of the spleen substance with alcohol, actually during the disintegration. A spleen was cut into two portions. One of these was minced in cold alcohol, which was poured into the mincing machine with the spleen substance. After thorough extraction, and removal of the alcohol, the extract was tested against acetylcholine chloride, and found to contain the activity-equivalent of 13 mg. of that substance per kg. of spleen. The other portion was minced and weighed before alcohol was added, the procedure occupying a few minutes only. The extraction and preparation for testing were thereafter carried out in strict parallel with those of the first portion. The activity-equivalent was only 0·75 mg. of acetylcholine chloride per kg. of the same spleen substance.

The fact that nearly the whole of the active principle thus disappeared from the minced tissue in the few minutes preceding the addition of alcohol, made it unlikely that, even with the greatest precautions, we should extract and preserve the whole of what was originally present. One point, however, was made clear, namely that disintegration of the spleen substance was the main cause of loss. Spleens kept intact for hours in the laboratory, in one case over night, yielded, on an average, extracts as rich in the unstable principle as those worked up immediately after death, provided that mixture with alcohol and mincing were simultaneous in every case. We were unable, further, to detect any loss when once the mixture with alcohol had been effected; the mixture of alcohol and mince could be left for days in the cold store before filtration, without detectable loss of activity. The yields from individual spleens, all extracted under the best conditions indicated by these trials, showed fairly wide variations. Expressed in terms of acetylcholine, the activity might be as low as 5 or as high as 30 mg. per kg. of spleen. It should be noted that the same range of activities would approximately correspond to those of 5 to 30 grammes of choline, acetylcholine being about 1000 times as active. The weakest of these extracts, made with the precaution mentioned from individual spleens, therefore contained substantially more of this activity per kg. of spleen than our original large-scale extract from ox spleens, in which the equivalent was 2 g. (= about 2 mg. of acetylcholine) per kg.

These experiments, while providing indications for large-scale procedure, had given additional evidence as to the nature of the substance with which we were dealing. It was not only unstable in alkaline solution, but was destroyed even more rapidly when liberated from the

spleen cells which presumably contained it, and brought into contact with the cells and juices of the whole minced organ. The behaviour was so strongly suggestive of a choline ester, resembling acetylcholine in its properties, that we decided to make a more complete comparison of its physiological actions with those of that substance.

By this time our preliminary chemical experiments had so far advanced as to show that the unstable principle was precipitated by mercuric acetate, and was recoverable from the precipitate. In an extract made with suitable precautions from a spleen, and purified in this manner, the choline-like effect so greatly preponderated in the depressor action, that any histamine still present made no significant contribution. A dilution of this purified extract was prepared which, on being tested for depressant action on a cat under ether, against a standard solution of acetylcholine, gave an equivalent of 0·4 c.c. of diluted extract to 0·01 mg. of acetylcholine (Fig. 1). The same solutions were then compared with respect to their stimulating action on the tone and rhythm of an isolated loop of rabbit's intestine, suspended in 100 c.c. of warm oxygenated Tyrode's solution. This preparation, as shown by one of us in an earlier investigation (Dale(7)), is a very sensitive and specific reagent for acetylcholine. It is practically unaffected by histamine in the quantities here in question, and under favourable conditions it will discriminate between two submaximal doses of acetylcholine differing by not more than 5 p.c. We have used it constantly in the numerous determinations of acetylcholine required in the further course of these investigations. In this preliminary experiment it gave precisely the same equivalent as that obtained for the depressor effect in the cat, 0·4 c.c. of the same spleen extract producing a rise in tone of intestinal muscle identical with that produced by 0·01 mg. of acetylcholine (Fig. 2).

Fig. 1. Comparison of purified spleen extract and acetylcholine solution; blood-pressure of cat under ether.

The correspondence of the action with that of acetylcholine in these two directions was striking. Both these effects, however, are of a type which that ester shares with muscarine and other "parasympathetic" stimulants. There is another group of actions, however, which, among

such substances, the choline esters alone produce, and acetylcholine with a peculiar intensity. The easiest of these to observe and measure

Fig. 2. Comparison of same two solutions on isolated jejunum of rabbit.

was the curious slow wave of contraction, produced in denervated mammalian voluntary muscle. (Cf. Frank, Nothmann and Hirsch-Kauffmann(8), Dale and Gasser(9).) The sciatic nerve of a cat was cut by one of us (H. H. D.), under ether anæsthesia and with aseptic precautions. Ten days later the cat was again anæsthetized with ether, and the gastrocnemius on the denervated side was dissected, the Achilles tendon being connected with an isometric lever, and the femur transfixed by a steel rod clamped to a rigid frame. The external iliac artery on the other side was isolated and clamped close to its aortic origin, and a cannula inserted, through which, on release of the clamp, injections could be made into the aorta near its bifurcation, so as to be carried directly

Fig. 3. Comparison of same two solutions, given by arterial injection into cat under ether, for effects in producing contraction-wave of denervated gastrocnemius.

by the blood-stream to the denervated leg. The internal iliac arteries were tied. Fig. 3 shows the effects produced by the injection of 0·4 c.c. of the dilution of spleen extract used in the previous comparisons, and of 0·01 mg. of acetylcholine, in this manner. The contractions are as similar as those which two identical injections of acetylcholine, given with the same interval, would produce. Such evidence made it clear that, whatever its exact chemical nature might prove to be, we were dealing with a choline ester, and with one so similar to acetylcholine in all its characters that we might adopt the latter as our physiological standard for controlling the effects of our procedure, and might safely plan our chemical manipulations on the assumption that the substance which we were endeavouring to isolate was, indeed, acetylcholine itself. The result, as shown in a later section, proved that this assumption was correct.

III. Isolation of histamine from ox spleen.

The method adopted was that which had already been successfully applied by Best, Dale, Dudley and Thorpe(1) to liver and lung, and by Thorpe(2) to muscle.

Immediately after the killing of the animals, 50 kg. ox spleen were collected at the slaughter-house, brought to the laboratory and minced into 94 p.c. alcohol (1·5 litres per kg. tissue) without delay. The mixture was stirred at frequent intervals during the first three hours, and then allowed to stand over night. It was then filtered, and the mince was re-extracted with 2 litres of 60 p.c. alcohol per kg. fresh tissue. After standing over night, this extract was filtered, and the filtrate was mixed with the first extract. The combined fluid was then acidified with sulphuric acid, 0·36 c.c. 50 p.c. (vol.) acid being added for each litre of extract. After standing for four hours, the liquid was filtered through paper-pulp, and concentrated *in vacuo*; the temperature of the liquid did not rise above 25° during the distillation. Volume of concentrate = 5·8 litres.

A small sample of this concentrate was centrifuged, and diluted 16 times. 0·25 c.c. of the diluted liquid was found to be equivalent to 0·0025 mg. histamine in lowering the blood-pressure of the unatropinized cat, while 0·55 c.c. was equivalent to 0·0025 mg. histamine in the atropinized cat.

0·55 c.c. of the diluted liquid, tested on the unatropinized cat, matched a solution containing 0·0025 mg. histamine + 0·7 mg. choline chloride.

From these tests the histamine content of the concentrate was estimated to be 0·42 g., and the "choline" content 116 g.

The concentrate was treated with basic lead acetate until no more precipitate formed, when it was filtered. From the filtrate the main excess of lead was removed as sulphate. After filtration the small amount of lead remaining was removed with hydrogen sulphide. The lead-free filtrate was concentrated to 2 litres.

Physiological assay estimated the histamine and choline chloride contents of this at 0·56 g. and 112 g., respectively. (For measuring of the apparent gain of histamine, see below.)

To the 2 litres concentrate, 115 c.c. sulphuric acid were added. 88 g. of potassium sulphate were precipitated by this treatment; the liquid, after filtering, was only faintly acid to Congo red, and was concentrated *in vacuo* to 900 c.c. to remove some of the free acetic acid present. After making it up to 1 litre with water, 1 litre of 10 p.c. sulphuric acid was added. Phosphotungstic acid was now added, 13·2 litres 25 p.c. solution in 5 p.c. sulphuric acid being required for complete precipitation. The precipitate was filtered off, washed with 500 c.c. of 1 p.c. phosphotungstic acid in 5 p.c. sulphuric acid, and then repeatedly extracted with 75 p.c. acetone until very little more went into solution. The combined acetone extracts were distilled *in vacuo* until most of the acetone had been removed, and the acetone-soluble phosphotungstate was then decomposed in the usual way with baryta solution. Excess of barium was removed from the filtrate with carbon dioxide, and the solution was concentrated *in vacuo* to 2·6 litres.

The physiological assay, made as before, estimated the histamine and choline chloride contents of this at 0·261 g. and 5·23 g. respectively. It will be seen that about 38 p.c. of the histamine and 95 p.c. of the "choline" activity were lost at this stage.

To this solution were added 30 c.c. sulphuric acid, making it strongly acid to litmus, but hardly acid to Congo red, and then 750 c.c. of 40 p.c. silver nitrate solution (sufficient to produce an immediate brown precipitate on adding barium hydroxide solution to a drop withdrawn for testing). The precipitate thus formed ("purine" fraction) was removed, and to the filtrate was added hot, saturated barium hydroxide solution, until the "histidine" fraction (tested in the usual manner with ammoniacal silver nitrate) was precipitated. The filtrate from this was saturated with barium hydroxide to precipitate the "arginine" fraction.

The "histidine" and "arginine" fractions were suspended in dilute

sulphuric acid and decomposed with hydrogen sulphide. Hydrogen sulphide was removed by aeration, and excess of sulphuric acid by barium carbonate, from the filtrate. The physiological assay of these two solutions indicated that the "histidine" fraction contained 0·0141 g. histamine and the "arginine" fraction 0·250 g. The "arginine" fraction, which contained practically all the histamine-like activity, was refractionated by the silver method, and gave small "purine" and "histidine" fractions, which had negligible activities, whilst the activity recovered in the "arginine" fraction represented 0·20 g. histamine.

This "arginine" fraction was concentrated *in vacuo* to about 100 c.c. A small amount of crystalline material (not examined) was removed, and the solution again evaporated until the volume was about 25 c.c. 25 g. powdered barium hydroxide crystals were then added to the liquid, and the resulting paste was dried in a vacuum desiccator over sulphuric acid. The mass was then powdered, and extracted with absolute alcohol in a Soxhlet thimble for three hours. After regrinding the mass, which had set to a hard solid, it was extracted for five hours and then re-ground and extracted for a further five hours. In this way three alcoholic extracts were obtained, to which water was added. The solutions contained a small amount of barium, which was removed as sulphate on careful neutralization of the alkaline solutions with dilute sulphuric acid. The total activity of the combined alcoholic extracts represented 0·108 g. histamine, determined physiologically.

The addition of sodium picrate to this solution (120 c.c.) threw down 2·61 g. of a picrate, crystallizing in dark yellow prisms, melting at about 200°, and practically devoid of physiological activity. The mother-liquor was concentrated and treated with a further quantity of sodium picrate solution; 1·4 g. of picrate were obtained, which on re-crystallization from 25 c.c. water yielded 0·237 g. of pale yellow crystals, m.p. 232–4° with decomposition, while the solution was still warm. After these had been filtered off, on cooling, the liquor deposited 0·798 g. of darker prismatic crystals, m.p. 198–200°. After two re-crystallizations of the pale yellow crystals, 0·1997 g. of a picrate, melting at 236–8°, was obtained. This picrate was identical in crystalline form and physiological activity with an authentic specimen of histamine dipicrate, and a mixed melting point determination showed no depression.

Analysis: 0·0430 g. picrate gave 0·0817 g. nitron picrate.

Picric acid: found	... ...	80·40 p.c.
„	calc. for $C_5H_9N_3(C_6H_3O_7N_3)_2$	80·48 p.c.

Summary of yields of histamine at the successive stages of fractionation, expressed as mg. histamine.

Original extract	Basic lead acetate filtrate	Phospho-tungstic acid precipitate	Arginine fraction	Alkaline extraction	Pure histamine (isolated as dipicrate)
420	560	261	200	108	40

The results are very similar to those obtained in our examination of extracts from other organs. As we have earlier pointed out, the physiological estimates have not a high order of accuracy; they are, of necessity, made on very small samples, the results given by which are multiplied by very large factors. There are, however, discrepancies too large to be accounted for in this manner. The apparent gain of histamine in the treatment with basic lead acetate is seen also in some of our earlier records from other organ extracts. It has been explained by Bauer's and Richards's observation (30), that acetates have a definite depressor, vaso-dilator action, which intensifies that due to histamine. The acetates are again eliminated in the phosphotungstic precipitation, so that the yield at this stage should be compared with the estimate on the original extract, showing an apparent loss of about 38 p.c. Some of this is probably genuine, and is readily intelligible when the bulk of the precipitate to be handled at this stage is considered. It must further be remembered, however, that the total depressor effect of the original extract on the atropinized animal involves components due to potassium ions, and possibly other unspecific agents, which are eliminated in fractionating the phosphotungstates.

The loss in the silver fractionation is small (23 p.c.) and could probably have been almost entirely eliminated by including the "histidine" as well as the "arginine" fraction. This, however, would have carried on to the next stage, at which the most difficult separation occurs, a quantity of other substances out of proportion to the small additional amount of histamine. It will be noted that in this case, as with other organ extracts, it is in the arginine fraction that most of the histamine is precipitated. Berlin (5) expected, from Ackermann's (10) account of its isolation from a putrefactive mixture, that any histamine present in his spleen extracts would be precipitated in the histidine fraction; not finding any of the active substance there, he wrongly concluded that it was not histamine.

As with other organ extracts, we encountered a serious loss on the extraction with alcohol of the free bases from the arginine fraction (alkaline extraction). It is the fact that there is yet no method available by which histamine can be extracted quantitatively from such a mixture.

The method used, though the most effective which we have found, always involves a substantial loss. The final yield of pure histamine dipicrate, from the bases so extracted, could have been substantially improved by repeated re-working of the picrate mother-liquors. It seemed to us, however, that the time and labour would be ill spent. We had already an ample quantity of the pure salt for complete identification, and, when our evidence from other organ extracts is put alongside this from the spleen extract, a sufficient justification for concluding that this also owes its histamine-like action to the presence of histamine itself.

IV. Isolation of acetylcholine from horse spleen.

A. General description of process.

Horse spleens were chosen for this purpose on account of the greater ease of obtaining them in quantity at a known interval after death of the animal. We had evidence of a similarly acting unstable principle in the spleen of the ox, and there is no reason for supposing that the yield from it would have been less under similar conditions.

Since the unstable active principle of the crude extracts had been shown to be similar, in its physiological activities and chemical behaviour towards dilute alkali, to acetylcholine, the method adopted for the fractionation of the extract was framed on the general lines of that employed by Ewins(11) for the isolation of acetylcholine from ergot; but numerous modifications were introduced, which seemed likely to increase the ease of operation and to conserve the very labile compound which we were attempting to isolate.

As in Ewins's method, the first stage was to get rid of everything from the extract which was insoluble in absolute alcohol; and it was found advantageous to proceed to this point without first carrying out the preliminary purification of the extract with mercuric chloride, which he had applied to ergot extract. The subsequent treatment of the material soluble in absolute alcohol was rather different from that of Ewins. He precipitated it directly as the mercury salt by the addition of alcoholic mercuric chloride, and extracted the active material from this precipitate with boiling water, then setting it free from its mercury compound by means of hydrogen sulphide, which, of course, produced a solution very strongly acid with hydrochloric acid. As acetylcholine salts are fairly easily hydrolyzed by boiling water, and also by mineral acids, it was feared that the application of these steps to the material from spleen might reduce seriously the already small amount present.

A gentler treatment was therefore devised. The alcoholic solution was diluted with an equal volume of water and the alcohol was then removed by distillation *in vacuo*. To the aqueous solution was added aqueous mercuric chloride and the precipitate produced, which contained none of the active substance, was removed. To the filtrate solid sodium acetate and powdered mercuric chloride were added; a heavy precipitate was formed, which, in a small exploratory experiment, contained the whole of the active material, of which 70 p.c. was recovered on decomposition of the precipitate with hydrogen sulphide[1]. Unfortunately, when this process was applied in the large-scale experiment reported in this paper, the separation of the active material was not so sharp, and successive treatments with mercuric acetate were necessary to remove a fair proportion of it from the solution. The step was therefore not such an improvement as was anticipated, and it might, after all, have been simpler to precipitate the mercury salt from alcoholic solution as Ewins did. The useful observation was made, that if the mercuric acetate precipitate was shaken with cold water a relatively small proportion dissolved, which contained the active constituent. A valuable purification was thus affected. The constituents soluble in cold water were treated with hydrogen sulphide at 0°, and the consequent development of acidity was not great.

At this stage Ewins got rid of a part of the relatively large amount of choline, which inevitably accompanies the active material in the fractionation, by converting it into the acid tartrate in alcoholic solution. Having crystallized out as much choline acid tartrate as possible, he precipitated the alcoholic filtrate with an alcoholic solution of platinum chloride, thus obtaining a mixture of chloroplatinates containing all the acetylcholine, together with excess of choline and other impurities. In the fractionation of the material from spleen this step was omitted. The aqueous solution from the decomposition of the soluble mercury salts was taken to dryness, and the portion soluble in alcohol was extracted from the residue. The alcoholic solution was then directly precipitated with alcoholic platinum chloride. This precipitate contained, by physiological estimation, about one-twentieth of its weight of acetylcholine chloroplatinate, the main impurity in it being choline chloroplatinate. A chemical investigation[2] of the problem of separating

[1] Partial or complete hydrolysis of acetylcholine occurs when its gold, platinum or mercury salts are decomposed with hydrogen sulphide (see Nothnagel (12)). Steps involving such decomposition must, therefore, be avoided as far as possible.

[2] This investigation will be described by one of us (H. W. D.) in a paper in the *Biochemical Journal*.

acetylcholine chloroplatinate from mixtures containing a large excess of the choline compound, showed that, from such mixtures, not acetylcholine chloroplatinate itself, but a double salt crystallizing in isotropic octahedra and containing one molecule of each (choline-acetylcholine dichloroplatinate) separated. The solubility in water of this salt, by means of which the acetylcholine from spleen was ultimately identified, is very much lowered in the presence of excess of choline chloroplatinate, which is itself very much more soluble in water than the double chloroplatinate. We had omitted the partial separation of choline and acetylcholine as acid tartrates, employed by Ewins. The omission was, at the time, dictated by caution, since the procedure involves the liberation of the free bases in alkaline solution, with inevitable loss of some of the ester; and the separation is, in any case, so incomplete (see Ewins(11)) that we doubted the advantage of attempting it. In the end, our neglect thus to reduce the excess of choline in the mixture proved to be a positive advantage, since, when the mixed bases had been converted into chloroplatinates, its presence depressed the solubility of the choline-acetylcholine dichloroplatinate, and enabled a substantial proportion of the latter to be finally isolated in pure condition. It was identified by analysis, by comparison with a pure sample prepared synthetically, and by determination of the physiological activity of the chlorides released from it, in comparison with that of the chlorides from the synthetic dichloroplatinate, and of acetylcholine chloride from its pure chloroplatinate.

The physiological method provided another test of the identity of this compound. If it was, indeed, a double chloroplatinate of choline and acetylcholine, it should yield on decomposition a solution containing 1 molecule of choline chloride for each molecule of acetylcholine chloride. Since acetylcholine is about one thousand times as active as choline, the choline would make no measurable contribution to the activity of such a mixture. If the latter, accordingly, was completely acetylated, the activity of the resulting product should be just double that of the original mixture. The change in activity could not, on the other hand, have this exact ratio, if the chloroplatinate under investigation were anything other than the dichloroplatinate of choline and acetylcholine. The test on the rabbit's intestine gave us an accuracy well within 10 p.c., and the experiment was made, first on the synthetic dichloroplatinate, and then on the supposedly identical substance obtained from the spleen extract. In both cases the result verified the prediction; complete acetylation exactly doubled the activity of the chloride-mixture on the rabbit's intestine.

B. Experimental details.

The spleens were collected from the slaughterers throughout the day, in batches of six. Killing commenced at 7 a.m., and the first batch of six spleens arrived at the laboratory at 10 a.m. Further batches of six arrived at 1.15 p.m., 5.0 p.m. and 8.15 p.m.

When the spleens arrived at the laboratory each was weighed separately; the weights varied between 1 and 2 kg. 96 p.c. alcohol was taken in the proportion of 5 litres alcohol per kg. spleen for each organ. The spleen was cut into three or four strips and immediately passed through a powerful mincer, one-tenth of its ration of alcohol being poured into the mincer as the tissue was passing through it; the mince was delivered directly into a small enamelled pail containing the remaining nine-tenths of the alcohol, which was vigorously stirred as the tissue fell into it. The mincing of each spleen was completed in about one minute. The alcoholic suspensions of minced tissue were bulked in larger containers, stirred frequently, and allowed to stand over night.

The total weight of the spleens so extracted was 32·34 kg. On the following day the extract was filtered, first through cloth and then through paper-pulp. The filtrate was then concentrated *in vacuo*; the temperature of the water jacket was held at 45–50°, and the temperature of the extract never reached 20°. It was reduced to a volume of 5 litres in the course of six hours.

The concentrated extract contained much finely divided fat. On shaking with ether a stiff emulsion was formed which was broken by the cautious addition of alcohol. The ether layer was removed and the aqueous layer, after another extraction with ether, was evaporated *in vacuo* to 1120 c.c.

Physiological assay of this solution, made on the rabbit's intestine, showed it to have an activity equivalent to 334 mg. acetylcholine chloride.

In order to remove some of the water and to salt out impurities, the solution was warmed to 26°, and anhydrous sodium sulphate was stirred in until no more would dissolve. Alcohol was then added until a copious precipitate of hydrated sodium sulphate was produced, which was filtered off after standing in the cold room. The filtrate was concentrated *in vacuo* to 400 c.c. More sodium sulphate was precipitated on adding an equal volume of alcohol and the filtrate from this was concentrated *in vacuo* to 150 c.c. The addition of alcohol to this concentrate caused a syrupy layer to be formed. It was therefore put in a

bottle with 2 litres absolute alcohol and shaken violently for two hours. The alcohol was poured off from a very sticky residue remaining on the sides of the bottle, and the extract was concentrated *in vacuo* to 200 c.c.

Physiological assays of the alcoholic solution and of the sticky residue showed activities equivalent to 260 mg. and < 10·5 mg., respectively, of acetylcholine. The residue was therefore discarded.

To the alcoholic solution 200 c.c. water were added, the alcohol was distilled off *in vacuo*, and to the residual solution (140 c.c.) 260 c.c. saturated mercuric chloride, and, finally, 30 c.c. of hot water containing 10 g. mercuric chloride were added. After standing in the cold room the precipitate was filtered off and washed with half-saturated mercuric chloride solution.

Physiological test of a solution prepared appropriately from the precipitate showed the latter to possess only a trace of activity.

To the filtrate were added 200 c.c. saturated aqueous mercuric chloride and then, cautiously, sodium hydroxide solution, since the filtrate was very strongly acid to litmus but not to Congo red. When the reaction to litmus was moderately acid the addition of sodium hydroxide was stopped and the precipitate which had formed was filtered off. This contained only a trace of activity. The filtrate was concentrated *in vacuo* to 170 c.c., and to it were then added solid sodium acetate and powdered mercuric chloride. The precipitate which formed was filtered off, and extracted repeatedly by shaking with successive quantities of about 200 c.c. water for 2-hour periods. The filtered solutions were immersed in ice-water and decomposed with hydrogen sulphide. After filtration from mercuric sulphide and removal of hydrogen sulphide by aeration, sodium hydroxide solution was added drop by drop until the reaction of the filtrates was faintly acid to litmus. They were then tested physiologically with the following results:

Water extract	Activity (as mg. acetylcholine chloride)
1	24
2	12·4
3	7·7
4	2·6

It was found that simple dilution of the solution, from which the mercury precipitate had been obtained, with saturated sodium acetate solution produced a further precipitate. An equal volume of the latter was, therefore, added; the precipitate produced was collected, extracted with water, and the extracts were decomposed and tested as before, with the following results:

Water extract	Activity (as mg. acetylcholine chloride)
1	31·4
2	7·2
3	0·5

The combined solutions, therefore, had a total activity representing 85·8 mg. acetylcholine chloride.

Physiological assay of the filtrate from which these two main mercury precipitates were separated detected an activity representing 36·8 mg. acetylcholine chloride, from which, by the addition of mercuric acetate and extraction of the precipitate with water as before, a solution of activity equal to that of 21·6 mg. acetylcholine chloride was obtained. This material was not added to the main bulk for further fractionation because it was heavily contaminated with sodium acetate.

The combined solutions of activity equal to 85·8 mg. acetylcholine chloride were concentrated to small bulk, the reaction was adjusted to faint acidity to litmus, alcohol was added, and precipitated salts were removed by filtration. The filtrate was then concentrated to small bulk and finally taken to dryness, over sulphuric acid, in a vacuum desiccator. The sticky residue was rubbed up with 24 c.c. absolute alcohol, and to the solution, after filtration from insoluble material, was added a solution of 3 g. platinum chloride in 15 c.c. absolute alcohol. The precipitated platinum salt was filtered off, washed with absolute alcohol and dried in a vacuum desiccator.

The weight of the platinum salt was 2·7183 g. A few milligrammes of this salt were decomposed by the potassium chloride method described by Ewins(11), and the activity, determined physiologically, referred to the total platinum salt, was equivalent to 88 mg. acetylcholine chloride. At this stage the platinum salt stood for 3½ months in a desiccator over calcium chloride, while the investigation of the properties of acetylcholine, already referred to, was being made.

On retesting after this period, its activity was found to correspond to 76·4 mg. acetylcholine chloride. A definite loss of activity had, therefore, occurred.

Isolation of choline-acetylcholine dichloroplatinate. The total weight of platinum salt used for the final stage of the isolation was 2·714 g. with an activity corresponding to 76·4 mg. acetylcholine chloride (equivalent to 0·1475 g. acetylcholine chloroplatinate or 0·2770 g. choline-acetylcholine dichloroplatinate).

The platinum salt was treated with 8 c.c. boiling water, which would have been sufficient to dissolve the material completely if it had been

simply a mixture of choline and acetylcholine chloroplatinates. As, however, complete solution was not effected, 50 c.c. cold water were added, and, after standing, 0·0486 g. (A) of an insoluble residue was removed by filtration. The solution was placed over sulphuric acid in a vacuum desiccator over night; the volume was thus reduced to 15 c.c., and octahedra similar in appearance to those of synthetic choline-acetylcholine dichloroplatinate, together with lighter coloured regular crystals, had been deposited. The solution was warmed, and, after the octahedra had gone into solution, decanted from the less soluble lighter material, 0·0244 g. (B). After cooling, the decanted liquid deposited octahedra which were filtered off; 0·378 g. (C). The filtrate was returned to the vacuum desiccator and reduced in volume to 7 c.c. It was warmed on the water-bath to dissolve the material which had separated, and, on cooling, deposited 0·070 g. of clean octahedra (D).

Weighed quantities of about 1 mg. each of (C) and (D) were decomposed by the potassium chloride method and tested physiologically against pure acetylcholine chloride. The result of the test was that, referred to choline-acetylcholine dichloroplatinate, both these fractions were about 50 p.c. pure.

(C) and (D) were, therefore, combined (0·4484 g.), and heated with 4 c.c. water on the water-bath. The whole of the material did not go into solution, as it would have done had it been pure choline-acetylcholine dichloroplatinate; 8 c.c. of cold water were therefore added, and, after 12 hours, the solution was filtered from a small amount of insoluble material (0·007 g.), and the solution was taken to dryness in a vacuum desiccator. The residue was again taken up in 4 c.c. hot water, but, as some material remained undissolved, the solution was held at 37° for 1½ hours, and the undissolved material was then filtered off; 0·138 g. (E). The filtrate, on cooling to room temperature, deposited octahedra, from which the solution was decanted after 5½ hours. The octahedra were washed with a little water, and weighed 0·0395 g. (F). The decanted liquid and washing were returned to the desiccator, and a further crop of octahedra removed when the volume was about 3 c.c.; 0·1030 g. (G). On concentrating to 1·5 c.c., as before, another crop of perfectly formed octahedra was obtained; 0·0914 g. (H). The final filtrate was taken to dryness, and, under the polarizing microscope, a small quantity of isotropic octahedra could be seen embedded in strongly anisotropic material (choline chloroplatinate).

The original solution from which (C) and (D) had been isolated was concentrated in a vacuum desiccator to about 3 c.c. The liquid was then

full of anisotropic prismatic needles (choline chloroplatinate). It was warmed until these needles were just dissolved, and then filtered to remove a small amount of minute regular crystals; 0·0220 g. (K). (A) and (B) were not examined, since their relative insolubility precluded the possibility of their being choline-acetylcholine dichloroplatinate.

Weighed quantities of (E), (F), (G), (H) and (K) were tested physiologically, after decomposition by the potassium chloride method, against pure acetylcholine chloride, with the following results:

	Taken (mg.)	Activity p.c.
(E)	2·5	2·45
(F)	0·95	52·2
(G)	0·8	48·5
(H)	1·15	55·1
(K)	0·8	7·1

The theoretical percentage activity of choline acetylcholine compared with that of acetylcholine, both weighed as chloroplatinates, is 53.

Within the limits of accuracy of the physiological method, the activities of the crops (F), (G) and (H) are, therefore, indistinguishable from that of pure choline-acetylcholine dichloroplatinate. The very small activities of (E) and (K) indicate that the fractional crystallization removed successfully a less soluble platinum salt, and that practically the whole of the acetylcholine was removed from the main solution in fractions (C) and (D).

Analysis: Two micro-analyses were made on material from crop (F).

	Found p.c.		Calculated p.c.
	1	2	
C	21·18	21·84	21·88
H	4·37	4·37	4·56
Pt	29·60	29·75	29·64

Melting points of crops (F), (G) and (H), synthetic choline-acetylcholine dichloroplatinate, and mixed melting points of each fraction with the synthetic salt were determined.

Material	Weight g.	Melting point °C.	Mixed melting point °C.
Synthetic choline-acetylcholine dichloroplatinate	—	260–1	—
(F)	0·0395	259–60	260–1
(G)	0·1030	259–60	260–1
(H)	0·0914	260–1	260–1
	0·2339		

The material isolated in crops (F), (G) and (H) is, therefore, identified as choline-acetylcholine dichloroplatinate. The total weight of the three crops was 0·2339 g. as compared with the physiological estimate of 0·2770 g. of the dichloroplatinate, as present in the crude mixture of chloroplatinates. It will be clear that a high proportion of the pure compound was separated from the crude mixture.

The amounts of acetylcholine chloride (in mg.) at each stage of the purification are set out in the following table. The estimates were made by the biological test, except in the last case, where the pure dichloroplatinate was weighed, and the corresponding amount of acetylcholine chloride calculated.

Original extract	Alcoholic solution	Hg acetate precipitate	Crude chloroplatinates	Crude chloroplatinates after standing 3½ months	Pure dichloroplatinate
334	260	85·8	88	76·4	64·5

It will be seen that the main losses occurred in the first two stages, the most serious loss being incurred in the precipitation with mercuric acetate and subsequent liberation of the substance from its mercury compound by means of hydrogen sulphide. Incomplete precipitation and unavoidable partial hydrolysis during decomposition of the mercury compound account for this fall in quantity. There was remarkably little loss after the mercuric acetate stage, and the yield of pure salt would undoubtedly have been higher if it had not been necessary to store the crude chloroplatinate for 3½ months while the chemistry of the final fractionation was being studied. Even so it is somewhat surprising, in view of the very unstable nature of acetylcholine, that about one-fifth of the amount estimated as present in the original extract was actually isolated as a chemically pure compound.

Acetylation of the mixed chlorides. 1·75 mg. of the dichloroplatinate from spleen was dissolved in 2 c.c. water; 0·2 c.c. of a solution containing 2 mg. potassium chloride was added, and the liquid was taken to dryness over sulphuric acid in a vacuum desiccator. The residue was extracted with four successive lots of absolute alcohol, each of 4 c.c., and the combined, filtered extract made up to 20 c.c. 10 c.c. of this solution were taken to dryness *in vacuo*, and the residue was dissolved in 10 c.c. water, giving a solution represented by *A* in the physiological records shown in Figs. 4 and 6. 5 c.c. of the solution were evaporated to dryness in a small test-tube immersed in a paraffin-bath at 95°. A tube drawn out to a capillary delivered the solution, a few drops at a time, at the bottom of the test-tube, so that the residue was deposited compactly. The evapora-

tion of the alcohol was facilitated by drawing air through a tube dipping about half way down the test-tube. 0·5 c.c. acetyl chloride was added to the dry residue; the tube was sealed and heated at 100° C. for five hours. The tube was then opened and placed in a flask. The excess of acetyl chloride was removed by vacuum distillation, 10 c.c. absolute alcohol were added, and, after standing for ten minutes to decompose traces of acetyl chloride, distilled off *in vacuo*. The residue was then dissolved in 10 c.c. water, giving a solution represented by *B* in Figs. 4 and 5. It will be noted that *B* represents only half the concentration of

Fig. 4.

Fig. 5.

Fig. 4. Tests, on two preparations of rabbit's jejunum, of solutions *A* and *B* (see text) in four different, submaximal doses. The equivalence is in all cases exact. *B* being made up to one-half the concentration of *A*, the activity has been exactly doubled by complete acetylation.

Fig. 5. Comparison of *B*, in equal doses, with pure acetylcholine solution *S* (see text).

original chloroplatinate represented in *A*. A solution of acetylcholine chloride was prepared from pure acetylcholine chloroplatinate corresponding to that contained in 1·75 mg. of the dichloroplatinate, and was similarly made up to 20 c.c. with water. This solution is represented as *S* in Figs. 5 and 6, showing its comparison with *B* and *A* respectively. It will be seen that it exactly matched these in activity.

The results of the test showed that the activity of the material, liberated from the dichloroplatinate isolated from spleen, was exactly

Fig. 6. Comparison of solutions *A* and *S*, in three different doses. Depressor effects on blood-pressure of completely pithed cat, maintained by slow, constant, intravenous infusion of adrenaline.

doubled by complete acetylation, and that the activity of the unacetylated material, in relation to that of pure acetylcholine chloride, had the theoretical value, viz. 53 p.c., on the basis of the acetylcholine content of choline-acetylcholine dichloroplatinate.

Discussion.

1. *Histamine.* The fact that histamine can be extracted by cold alcohol from the substance of the fresh spleen hardly needs separate discussion from that already given to similar findings in the case of other organs. In this, as in other cases, we regard the evidence as justifying the assumption that histamine is present in the cells of the living organ. With regard to its richness in this base, the spleen tissue seems to occupy an intermediate position. It contains less than the lung, and more than most other organs per unit of tissue. The physiological estimate on our large-scale extract showed about 8 mg. per kg. of spleen. This probably represents a higher concentration in the intrinsic spleen cells, since a substantial contribution to the weight of the whole organ must be made by the blood corpuscles, which contain very little histamine when they are in circulation. Our main concern was to show that there is no ground for suggesting that the histamine-like actions of spleen extracts are due to a special principle, or specific hormone. There is no real reason for attributing them to anything other than histamine itself.

2. *Acetylcholine.* The isolation of acetylcholine has an interest of a very different kind, this being the first occasion on which the substance

has been found to occur naturally in the animal body. It was first prepared artificially by Baeyer(13) in 1867, and had only a chemical interest until 1909, when Hunt and Taveau(14) observed that, though it resembled choline in the type of its depressor action, it was enormously more active. The interest in it of one of us (Dale(7)) was aroused by its identification by Ewins(11), then collaborating with him, as a natural constituent of certain ergot extracts. An examination of its action on various organs showed that it reproduced the effects of parasympathetic nerves, in an intense and evanescent form, with a fidelity rivalling that with which adrenaline reproduces effects of true sympathetic nerves. When these parasympathetic effects were annulled by atropine, acetylcholine, in somewhat larger doses, was found to possess another type of action, stimulating ganglion cells in a manner which ranged its action with that of nicotine. These two distinct types of activity could be traced, with varying intensities, in a series of other choline esters, other quarternary ammonium bases, and, as Hunt and Renshaw(15) have shown, also in phosphonium, arsonium, stibonium and sulphonium bases. Seen with peculiar intensity in the effects of acetylcholine, these "muscarine" and "nicotine" actions must be regarded as related to different portions or aspects of the structure of its molecule. One of its actions, to which much interest has attached in recent years, is its excitation of a peculiar, slow contraction, or wave of tonus, in certain normal muscles of frogs and birds, and in mammalian muscles deprived, by degenerative section, of their motor nerve-supply. (See Riesser(16), Frank, Nothmann and Hirsch-Kauffmann(8), Dale and Gasser(9).) There was a tendency to attribute this effect to the presence of a hypothetical, secondary, parasympathetic nerve-supply to skeletal muscle. Dale and Gasser, however, were able to show, by tracing the effect through a series of compounds, that it was essentially an aspect of the "nicotine," and not of the "muscarine" action.

The discovery of activities of such interest, in a compound closely related to so general a tissue constituent as choline, has naturally provoked speculation as to the possibility that acetylcholine might occur in the body and be physiologically functional. Le Heux(17), taking as a basis Magnus's conception of choline as the normal stimulant of intestinal rhythm, observed that the action of choline on an isolated loop of intestine was enhanced by the presence of acetates in the surrounding saline medium, and speculated on the possible synthesis of acetylcholine as a preliminary to the effective action of choline. Hoet(18) observed that the atropine-sensitive rhythm of isolated intestinal muscle

was enhanced by strong, post-mortem faradization of the vagus nerves, before excision of the intestine. Hess(19), who has worked particularly on the slow contraction of frog's voluntary muscle in response to acetylcholine, detected in Ringer's solution, flowing from the perfused muscle during tetanic excitation, traces of a substance having activity suggesting that of acetylcholine. By far the most suggestive indications, however, of the natural function of a substance of this nature in the body have been afforded by the demonstration that the inhibition of the heart muscle, by stimulation of the vagus, is associated with, and presumably due to, the liberation of such a substance in intimate relation to the heart muscle fibres. Preliminary observations of this nature were made by Dixon(20) as long ago as 1906, before the activity of acetylcholine had been discovered. In a relatively recent series of investigations on the liberation of substances transmitting the effects of nerve stimulation in the frog's heart, Loewi and his coworkers(21) have produced an array of highly suggestive facts bearing on the nature of the "vagus substance." None of these facts has conflicted with the possibility of this substance being acetylcholine, and some of them clearly indicate a choline ester of similar properties.

The facts above mentioned, as to the dual nature of the action of acetylcholine, are also highly suggestive, when considered in relation to reactions of denervated voluntary muscle. Vulpian and Philippeaux(22), in 1863, showed that, after degenerative section of the hypoglossal nerve, the voluntary muscles of the tongue acquired a new type of sensitiveness, the organ responding with a slow type of contraction to peripheral stimulation of the chordalingual nerve, or of the separated *chorda tympani*, which normally produces only the well-known parasympathetic vaso-dilatation and secretory activity. Heidenhain(23), who later studied this phenomenon and termed it a "pseudomotor" reaction, endeavouring to explain it as due to turgescence with lymph, added the observation that a similar reaction of the denervated tongue was produced by nicotine. The phenomenon has received further study from v. Rijnberk(24) and others. More recently Frank, Nothmann and Hirsch-Kauffmann(8) observed that a closely similar reaction of the denervated tongue was produced by intravascular injection of acetylcholine. We have, then, this curious association of facts, that the stimulation of a parasympathetic nerve and the injection of acetylcholine, the action of which in the normal organ closely simulates the effect of such stimulation, both produce in the denervated organ a new reaction of the voluntary muscle fibres. If we suppose, on the analogy

of Loewi's experiments on the heart vagus, that parasympathetic nerves produce their effects by peripheral liberation of a chemical stimulant, we must also credit this substance with a stimulant action on denervated voluntary muscle; it must, therefore, be a substance having the two types of activity which are exhibited by the choline esters, and by acetylcholine with unique intensity.

We meet a similar relation, between normal vaso-dilator action and abnormal stimulant action on denervated voluntary muscle, in the phenomenon described by Sherrington(25) in 1894. He found that, when the muscles of the hind limb had been deprived of their motor innervation, by degeneration following spinal root section, stimulation of the sciatic nerve caused an abnormal, slow, weak contraction of the leg-muscles. The presumption was that the effect was produced by antidromic stimulation of the sensory nerve fibres. The only other possibility was an action of the sympathetic fibres, and this was excluded by v. Rijnberk(26), who produced Sherrington's phenomenon after preliminary degeneration of these. Hinsey and Gasser(27) have given further precision to the identification, by showing that the abnormal contraction of the voluntary muscles, and the normal antidromic vaso-dilatation, are alike produced by the sensory fibres of small diameter in the sciatic. In this case, again, we have a concurrent acquisition, by the muscle fibres deprived of their motor innervation, of a new sensitiveness to acetylcholine on the one hand, and to the stimulation of nerve fibres which normally cause dilatation of arterioles on the other. Hunt (28) has shown that the distribution of the vaso-dilator action of acetylcholine in the hind limb is closely similar to that of the antidromic vaso-dilatation.

We have again two sets of facts, which are at once brought into an intelligible relationship, if we make for the antidromic vaso-dilator effects the same supposition as for parasympathetic effects, namely that they are mediated by the peripheral liberation of a substance which dilates normal arteries and stimulates denervated voluntary muscle—of a substance which has the dual action of acetylcholine.

The suggestion, that these paradoxical responses of denervated voluntary muscle might find their explanation in the intervention of such a chemical mechanism, has been made by Bremer and Rylant(29), and more recently, and in more definite terms, by Hinsey and Gasser(27). But there has been a natural and proper reluctance to assume, in default of chemical evidence, that the chemical agent concerned in these effects, or in the humoral transmission of vagus action, was a substance known,

hitherto, only as a synthetic curiosity, or as an occasional constituent of certain plant extracts. Many things could be explained if the liberation of acetylcholine could be postulated; but the minuteness of the quantities required to produce the effects in question, and the extreme instability of the substance, while enhancing its theoretical fitness for the suggested functions, precluded any hope of its chemical identification at the sites of its possible liberation. There are certain discrepancies between the effects of atropine on the vaso-dilator effects produced by acetylcholine and by stimulation of parasympathetic and sensory nerves, and also between the antagonisms of adrenaline to these two methods of exciting the contraction of denervated muscle; but discussion of these may, with advantage, be deferred to a later communication, directly dealing with some of them.

It appears to us that the case for acetylcholine as a physiological agent is now materially strengthened by the fact that we have been able to isolate it from an animal organ and thus to show that it is a natural constituent of the body. We have yet no conception of the meaning of its presence in the spleens of these large ungulates. In a few quite preliminary experiments we have failed to find evidence of its presence in the spleens of the animals ordinarily available for the supply of fresh post-mortem material in the laboratory—dog, cat, monkey, or rabbit. In those of the ox and the horse, after our first accidental observation of its presence, we found it regularly, and in such quantities as to make the attempt at its isolation a reasonable and, in the event, a successful enterprise. The peculiar difficulties of dealing with a substance so labile make it very unlikely that we can extend the attempt to a range of other organs, as we have in the case of histamine. We feel, however, that its definite isolation from one organ has so far altered the position that, when an extract from, or a fluid in contact with the cells of, an animal organ can be shown to contain a principle having the actions, and the peculiar instability, of acetylcholine, it will be reasonable in future to assume the identification. On such lines a physiological survey of its distribution should be practicable. Similarly, when there is evidence associating some physiological event with the liberation of a substance indistinguishable from acetylcholine by its action, the presumption that it is, indeed, that ester will be strengthened by the knowledge that acetylcholine occurs in the normal body. That is the only significance which we can, as yet, attribute to its identification in the horse's spleen, though further evidence may associate its occurrence there with some function yet unknown.

SUMMARY.

1. The substance in alcoholic extracts of spleen, resembling histamine in its action, has been isolated and identified as histamine.

2. Treatment of the fresh spleen substance of the horse or ox, with alcohol applied simultaneously with the disintegration of the organ, extracts an intensely active, unstable ester of choline, which has been isolated from horse's spleen and identified as acetylcholine.

3. The bearing of the latter identification on certain physiological phenomena is discussed.

At various stages of this investigation we have been greatly indebted for help, especially in making the numerous physiological estimates involved, to Drs Bauer, Richards and Gaddum, and to Mr Collison of the laboratory staff. To Mr Marks, in addition to co-operation already acknowledged, we are indebted for the analyses by micro-combustion, and for much valuable assistance in the large-scale extractions.

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THE RESPONSE OF SMOOTH MUSCLE TO STIMULATION OF THE VAGUS NERVE.

By B. A. McSWINEY and J. M. ROBSON.

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A PRELIMINARY description of a vagus nerve smooth muscle preparation was recently given by one of us(1). This paper deals with further experiments using the same preparation. Little or no literature is available on the response of isolated smooth muscle to indirect stimulation, though Snyder and Light(2) have described a preparation in their experiments on the heat production of the urinary bladder of terrapin.

Method. Rabbits, cats and dogs were used in these experiments. Cats and dogs were deprived of food for at least 12 hours, and then given a meal of meat juice 3 hours before the experiment. Rabbits were killed by a blow on the occiput, while cats and dogs were anæsthetized with ether. The stomach and œsophagus were removed from the animals and dissected under warm Ringer-Locke solution. The vagus nerve was identified on one side and dissected free from underlying tissue. The muscle supplied by the nerve was separated from the mucous membrane and removed as a strip. After some 20 minutes in warm solution the muscle was suspended in Ringer-Locke or Tyrode solution. Experiments were carried out at 37° C., bubbling and hydrogen-ion concentration being regulated.

Optical methods were used to record the response of the muscle. A small galvanometer mirror was attached to a torsion spring and the movements of the beam of light recorded on bromide paper. Tracings were also made on smoked paper with light balanced levers. An induction coil was used, the nerve being placed on platinum electrodes outside the chamber. The frequency of the coil for tetanizing currents was 50 make and 50 break shocks per sec.

Experimental results. Strips of cross-striated muscle supplied by the vagus nerve were removed from the rabbit's or dog's œsophagus and the upper region of the cat's œsophagus. The muscle usually responded by a quick twitch on stimulation of the nerve with a single break induc-

tion shock. With a tetanus the contraction was sustained, but gradually diminished in height as stimulation was continued.

Fig. 1. Isometric response of gastric smooth muscle to stimulation of vagus nerve with single break induction shock. Time interval: 0·04 sec.

Smooth muscle preparations were dissected from the lower end of the cat's œsophagus, the fundus and upper body of the stomach. On stimulation of the vagus nerve with a single break induction shock the muscle contracted. The tracing showed a long latent period, a gradual contraction and a corresponding long relaxation phase. The latent period, while varying in different preparations, remained constant throughout the same experiment, provided fatigue was prevented. The results are expressed in Table I.

TABLE I.

Tissue	Latent periods (sec.) Average	Longest	Shortest	Number of observations	Stimulation
Circ. œsophagus	0·52	0·7	0·4	8	Ind. shock
,,	0·55	0·75	0·38	33	Tetanus
Long. œsophagus	0·47	0·52	0·39	7	,,
Stomach	0·77	1·2	0·58	41	Ind. shock
,,	0·7	0·75	0·65	6	Tetanus

The average latent period obtained with a preparation of the gastric musculature was 0·77 sec., which is appreciably longer than the average latent period of the œsophagus. The response was slow and gradual, yet the onset of contraction was easily distinguished on the record. The duration of the rising phase of contraction appeared to depend on the condition of the preparation, varying between 1·7 and 2·7 sec.

TABLE II. Stomach: break induction shock.

	Average (sec.)	Longest (sec.)	Shortest (sec.)
Contraction	2·2	2·7	1·7
Relaxation	6·2	7·2	4·2
Half relaxation	2·8	3·4	2·0

Relaxation was more prolonged than contraction. Half relaxation time averaged 2·8 sec., while total relaxation usually took some 6 to

8 sec. It was possible for an hour to obtain a response to a single shock; after that the muscle would only respond to a tetanus.

The response of different smooth muscle preparations to a tetanus was found to vary considerably. With the circular muscle of the lower region of the cat's œsophagus relaxation commenced soon after the maximal height of contraction had been reached, even though stimulation was continued. Occasionally a second response, or a series of contractions, was observed. With longitudinal preparations from the lower end of the œsophagus it was usual on the other hand to obtain a sustained contraction with small superimposed waves with continued stimulation, the muscle remaining in contraction for some 30 sec. or more before relaxation took place. Occasionally the response was a series of waves, but this effect was associated with a long latent period and a small contraction which suggested fatigue or damage of the preparation.

Strips from the gastric musculature with tetanus showed a sustained contraction with small superimposed waves, the contraction being always greater than the response to a single shock. The rise to maximum tension usually took 10 or more seconds. Relaxation during stimulation only occurred if the latter were continued over a long period, but on cessation of the stimulation the muscle relaxed after a short latent period. Occasionally rhythmic contractions occurred during prolonged stimulation when the relaxation phase was considerably delayed, or a further contraction was recorded on cessation of the stimulation. Similar results have been obtained from the preparation of the dog's stomach, though all phases of the response were longer than those described for the cat. Experiments have also been made with lower frequencies, using a Lewis contact breaker, and sustained contractions have been obtained with 5 make and break shocks per sec.

The effect of two shocks was investigated, the second being timed to fall at varying intervals after the first. For intervals up to 0·03 sec. a Lucas contact breaker was used, while for longer intervals a special instrument was devised. It consists of an oil dash pot with an arm mounted on the top of the piston. This arm was arranged to knock over two break keys of the Lucas type in its fall. The distance between the two keys and the rate of fall of the piston may be varied.

The nerve-muscle preparation was made from a circular strip of the cat's fundus. After the preparation had reached a steady state the vagus nerve was stimulated with maximal break shocks and the contraction recorded. The second stimulus was timed to fall very soon after the first,

and with an interval of 0·005 sec. a larger contraction was obtained than with one break shock. As the interval between the two stimuli was

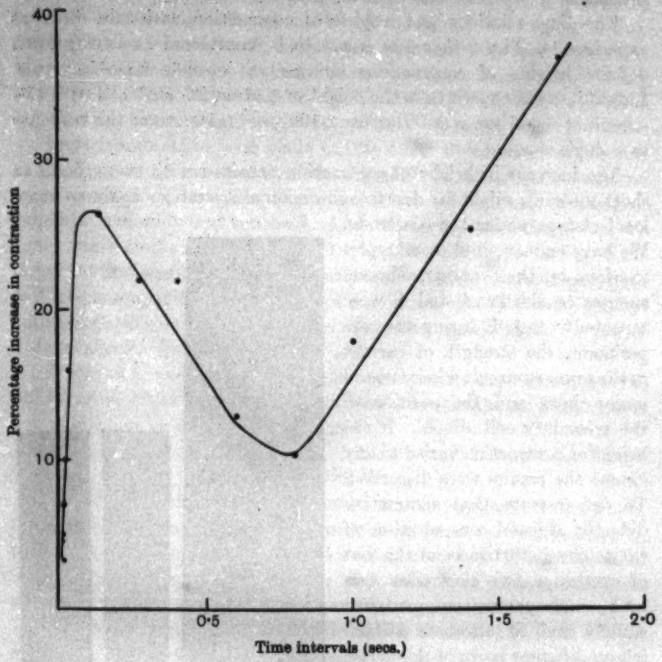

Fig. 2.

increased the height of contraction became greater until, as shown in Fig. 2, the maximum response was obtained with an interval of approximately 0·1 to 0·15 sec. The contraction diminished with further increase in the interval until, with a period of 0·8 sec., the second stimulus had little effect. With further increase in the interval the effect of the second stimulus increased again. With an interval between the two shocks so adjusted that the second stimulus fell towards the end of the latent period, or during the commencement of contraction, two stimuli gave a larger or summated contraction; with a greater interval, however, two

distinct responses were apparent on the record. The latter part of the curve shown in Fig. 2 therefore was due to a true muscular summation.

The time relations and heights of contraction varied in different experiments. Fig. 2 therefore is not to be considered as setting forth definite heights of contractions obtained at specific time intervals. Indeed in some experiments the height of contraction obtained with two stimuli at 0·025 sec. was 50 or even 100 p.c. greater than the response to a single shock.

The increase in height of contraction obtained with two stimuli at short intervals might be due to sub-maximal stimuli or again to some local change under the electrode. We have endeavoured to take precautions on these points. The responses to shocks *A* and *B* were repeatedly tested during the experiment, the strength of current in the primary circuit was increased many times and the position of the secondary coil altered. If the height of contraction varied to any extent the results were discarded. To demonstrate that summation did not depend on addition of excitatory disturbances at the seat of excitation two electrodes were used, and summation occurred equally well if alternate stimuli fell on different parts of the nerve. Observations have also been made on the effects of change in hydrogen-ion concentration. Starting with the solution at *p*H 8, the *p*H of the solution was gradually decreased. At various points the effect of two stimuli, separated by an interval of 0·025 sec., has been tested. The results show that again two stimuli were more effective than one. On decreasing the *p*H the muscle may fail to respond to a

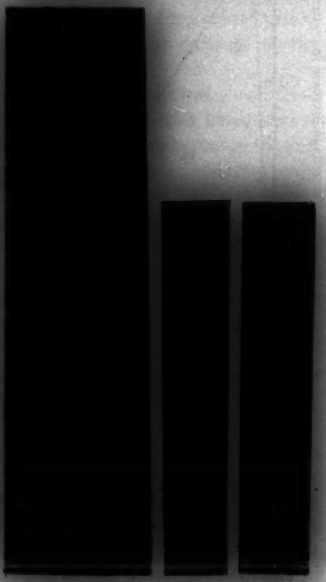

Fig. 3. Tracing to show effect of atropine. Isotonic lever. *A*. Normal response to tetanus. *B*. Addition of atropine. *C* and *D*. Response to tetanus. Time interval: 10 sec.

single stimulus, yet react to two successive stimuli. Again, at low *p*H values, the muscle may only contract to a tetanus.

After the tissue has been suspended in the bath for a period its height of contraction decreases and it fails to respond to a single stimulus. For a short time two stimuli may effect a response if the second stimulus is timed to fall 0·03 sec. or later after the first. Later, with further impairment, three or even four stimuli are required, and finally the muscle will only respond to repeated stimulation of the nerve.

Experiments have been made on the effect of addition of atropine as the drug is known to paralyse the vagus fibres. After contraction had been recorded on tetanizing the vagus nerve 0·1 to 1 mg. of atropine sulphate was added to the bath. After a short period the nerve was again stimulated and a well-marked relaxation obtained. In Fig. 3 the muscle at first was stimulated indirectly with currents of varying strength and frequency, and in every instance a contraction was obtained. After addition of atropine at *B* the muscle relaxed on tetanizing, or even after a single shock.

Ergotoxine, even though added to the bath in large amounts, was not found to affect the contraction obtained on stimulation of the vagus nerve with one or more induction shocks.

Discussion.

Preparations of mammalian smooth muscle supplied by the vagus nerve have been made and the response of the muscle on stimulation of the nerve with single and repeated stimuli recorded. The duration of latent periods, and of contraction and relaxation phases, has been determined and is remarkably constant if fatigue be prevented.

The larger contraction obtained with a tetanus and two stimuli separated by short time intervals is, in our opinion, explained on the basis of peripheral summation. The experimental evidence is against local addition of excitatory effects in the nerve fibre and in favour of summation of the effect of stimuli in the peripheral nervous network. In the first place, summation was obtained equally well if alternate stimuli fell on different parts of the nerve fibre. Again the same result was observed with short and relatively long time intervals. Moreover, the observations made at high hydrogen-ion concentrations and with fatigued preparations agree with the findings of other observers.

Summation of stimuli has been described in similar tissues, as Engelmann(3) showed that, in the ureter, electrical stimuli which failed to produce any contraction when acting singly were successful

when repeated at intervals of half a second. Romanes(4) made similar observations on the umbrella of the medusæ, and Richet(5) has given an account of phenomena observed when stimuli are applied to the motor nerve of the claw muscles of the crayfish. Lucas(6) confirmed Richet's observations and showed that peripheral summation could be observed in the frog's motor nerve after prolonged fatigue of the preparation. Lucas(7) suggested that summation depended on the development of a supernormal period after the refractory period is over.

Peripheral summation, as observed in our experiments, is probably due to the presence of the peripheral nervous network known as Auerbach's plexus. The cells of Auerbach's plexus must, according to Gaskell(8), be regarded as "the motor nerve cells from which arise motor nerves to that part of the involuntary intestinal musculature which is associated with the vagus portion of the cranial outflow of connector nerves." This plexus has the properties of a synaptic system and therefore possesses all the conditions necessary for peripheral summation.

Summation of contraction has been recorded with two stimuli separated by an interval greater than 0·8 sec. With a tetanus a sustained contraction was obtained in some preparations, relaxation occurring rapidly on cessation of stimulation. Tetanus of smooth muscle has been previously described on direct stimulation, as Howell(9) states that the stomach muscle of the frog requires only one stimulus each five seconds to cause tetanus. No "angle" as obtained with striated muscle was distinguishable on any of the smooth muscle records.

Summary.

An isolated vagus nerve smooth muscle preparation has been obtained. The muscle contracted on stimulation of the nerve with a single break induction shock and tetanus. The average latent period of gastric smooth muscle equals 0·77 sec., contraction 2·2 sec., and relaxation 6·2 sec. Peripheral summation of stimuli has been demonstrated, and summation of contractions was recorded with two or more stimuli. The contraction evoked by stimulation of the nerve was converted into relaxation by the addition of atropine.

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A METHOD OF RECORDING REFLEX MUSCULAR CONTRACTIONS IN MAN[1].

By E. A. BLAKE PRITCHARD.

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THE apparatus here described was devised in the attempt to obtain from intact human muscle in living subjects contraction records comparable with those obtained from experimental animals whose muscles may be freed from their insertions and attached to some recording lever.

Contraction curves from human muscle have previously been obtained either by recording changes in thickness of the muscle or in the position of the limb segments upon which the muscle acts. The complex structure of the muscles used and their disposition in the body allow of no accurate correlation between thickening and shortening records either in their general form or in their time relations. In those obtained by leading from some segment of the limb acted upon by the contracting muscle, the additional factors introduced by the inertia and momentum of the part moved, the unknown participation of the other muscles acting upon the same part, especially of the physiological antagonists, and the necessity for the conversion of an angular into a linear movement are all difficulties which prevent the resulting curves from being comparable with the direct shortening curves of detached muscles.

It was felt that if some means could be adopted of leading from the tendon of a muscle which was free to contract or relax without producing any change in the disposition of the body in which it lay, suitable records might be obtained, and the occurrence in the tendon of the quadriceps femoris muscle of a sesamoid bone—the patella—offered the opportunity sought. When the leg is fixed in flexion at the hip and full extension at the knee this muscle is free to contract and relax opposed only by the forces of:

Interfascial friction in the tissues surrounding the muscles;

The inertia of the patella;

[1] Work done with the assistance of a grant from the Medical Research Council.

Gravity in so far as contraction or thickening entails any raising of the centre of gravity of the muscle.

The movements of the patella can be recorded as those of a bar rigidly fixed to it—either by transfixing it by sharp points through the anæsthetized skin, or since the skin is here sufficiently lax with the knee in extension to move along with the underlying bone, by fixing such a bar firmly to the patella through the overlying skin. The applicability of this second method varies from person to person according to the thickness of the subcutaneous fat, but in most cases there is every indication from the results obtained that the bar follows with sufficient fidelity the movement of the bone and tendon to which it is attached. Since the patella may also be pulled down the leg towards the foot by a force applied to the apparatus attached to it, any required degree of tension can be established in the muscle and variations in tension during contraction, if they occur, can be measured.

Description of the apparatus. The subject sits upon a substantially built table clamped to a bar bolted through the wall. He reclines against an adjustable back-rest and extends both legs along the table. The leg from which records are to be obtained is strapped firmly to a padded back splint, *S* (Fig. 1), which is in turn bolted through the table in such

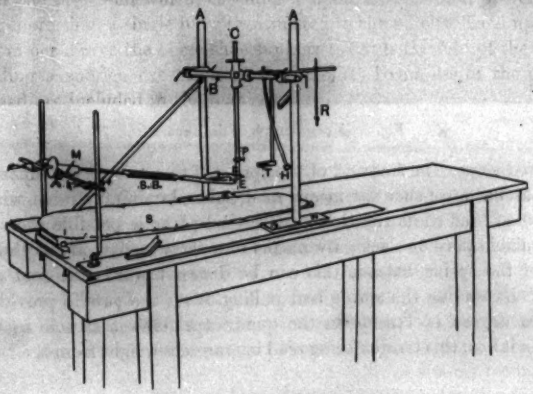

Fig. 1. A diagram of the apparatus.

a manner as to allow of its being raised and lowered but not moved in the axis of the limb. Two rigid strutted, vertical pillars, *A*, one on either

side of the table, carry a horizontal member, *B*, upon which is hinged a vertical bar, *C*, free to swing in the direction of the table's length. This bar is extensible and its length controlled by a screw collar, *D*, at the upper end. At its lower end it carries a piece of iron which is shaped like an under-cut horse-shoe, *E*, the lower edge of which can be made to engage behind the upper border of the patella.

At the foot of the table another horizontal bar, supported at any convenient height upon two vertical pillars, carries a rod terminating in a spring balance, *SB*, provided with a clamp for fixing it at any

Fig. 2. A photograph of the apparatus.

required slope. The free end of the spring of this balance is connected to the patella horse-shoe by means of a light aluminium frame which is hinged so that while rotation in a vertical plane is possible there is no appreciable play or slack. By means of a screw collar, *M*, at the fixed end of the spring balance this can be drawn towards the foot of the table. Extending the spring and pulling down the patella provide any desired degree of tension in the quadriceps femoris muscle in whose tendon it lies; this tension being read in grammes weight from the balance scale.

If the muscle is stimulated to contract under the above conditions the patella is pulled towards the head of the table against the tension of the spring, and the vertical bar carrying the horse-shoe-shaped member rotates about the horizontal axis of its hinge.

The movements of this bar, or rather of a fine needle soldered rigidly parallel with it towards its lower end, are recorded upon the falling plate of a kymograph camera which receives the shadow thrown by this needle in a field of illumination provided by a Pointolite lamp whose rays are first converged to a point by means of a system of spherical lenses and, thence diverging, are passed through a hemi-cylindrical lens which concentrates the beam in the plane of the camera aperture.

The contractions of the quadriceps in response to any stimulus, voluntary, involuntary, electrical, mechanical, etc. may be in this way recorded.

It is the contraction in response to sudden stretch (the suprapatellar or knee-jerk) that has been particularly studied by means of this apparatus, and to provide the necessary increment of tension or of length in the muscle a rubber-faced hammer, *H*, carried by a laminar spring was arranged to strike upon a circular metal plate, *P*, clamped to the bar whose movements were recorded. The upper end of this spring is movable along a horizontal support hinged at the front end and movable in a vertical plane. This carries also a catch, *R*, for holding the spring in any degree of deformation and releasing it at the right moment, and a ratchet, *T*, which is knocked over as the spring passes on its way to deliver the blow and which, catching it upon the rebound, prevents its giving more than a single blow transmitted to the patella. The impact of the hammer drives the horse-shoe support towards the foot of the table, providing a sudden extension of the muscle. Examples of the curves obtained are included in the text.

These show that whether the normal muscle be led to contract by electrical stimulation over the motor point or reflexly in response to a sudden stretch, the curve obtained is characterized by a period of contraction and a plateau of maintained shortening which terminate in an angle preceding the period of relaxation. That the shape of these curves may be greatly altered in different circumstances, especially in diseases of the nervous system, is also shown in the examples submitted.

In records previously obtained by other workers of the shortening reactions to sudden stretch, *i.e.* of the knee-jerk, in man the form of the muscular contraction did not reveal those elements of the curve shown by Sherrington and others to be characteristic of similar records obtained from animals. This was probably due to a smoothing out of the curve by the inertia and momentum of the moving parts and to the fact that the action of other muscles had not been excluded. In the records here given these elements, the rectilinear ascent, the horizontal

plateau and the "angle" preceding relaxation, are all clearly demonstrable and are in man subject clinically to the same modifications as have been found with comparable lesions of the central nervous system artificially produced in animals.

Measurement of the curves. The *initial tension* is read from the scale of the spring balance. The spring most often used extended 13 cm. between 0 and 1000 g. The maximum excursion of the patella from full relaxation to full voluntary contraction of the muscle is less than 1·4 cm. All recorded contractions have a recorded excursion of less than this distance, the average being 1·0 cm., so that the muscle does not contract isotonically but is progressively loaded during contraction by an increase of weight of up to 70 g., which represents a relative increase in the load of 7 p.c. to 15 p.c. A much greater degree of isotonicity may be established by using suitable springs.

The stimulus. When the muscle is stimulated to contract reflexly in response to sudden stretch the amount of lengthening can be measured directly from the curve obtained. This figure is of more importance than the absolute energy of the blow delivered. The amount of lengthening is directly proportional to the shift of the shadow consequent upon the blow, *i.e.* the dip in the curve. The increase of tension produced by such lengthening can be found subsequently by progressive loading of the muscle with definite increments of tension, a part of the plate being exposed at each step.

Summary.

There has been described a piece of apparatus and the method of its use for obtaining shortening records of muscle contractions in living, human subjects.

The advantages claimed for this method are:

1. The smallness of the inertia and momentum of the moving system.

2. The functional isolation of the muscle recorded independently of changes in any other muscles.

3. The ease with which the tension in the muscle, supplied through its natural attachment and in the natural direction, may be adjusted and determined.

4. The possibility of recording the shortening of the muscle directly.

It is held to improve upon those previously employed in giving a much more accurate record of the actual shortening reaction and one

which demonstrates clearly those features shown by Sherrington and others to be characteristic of similar contractions in animals[1].

EXAMPLES OF PHOTOGRAPHIC RECORDS OBTAINED (FIGS. 3–9).

The record should be read in each case from right to left. The time-marker (below) indicates in each case 1/100 sec. The preliminary dip in the curve corresponds to the passive extension due to the blow in response to which the knee-jerk occurred.

Fig. 3. A normal knee-jerk.

Fig. 4. Knee-jerk from a case of spastic paraplegia.

Fig. 5. Knee-jerk from a case of spastic paraplegia: more slowly falling plate.

[1] Reference to results obtained by this method of recording the knee-jerk, and by others, is made in a forthcoming paper in *Brain*, 1929.

Fig. 6. A record of spontaneous clonus occurring in a patient with spastic paraplegia.

Fig. 7. Knee-jerk from a patient with cerebellar hypotonus.

Fig. 8. Knee-jerk from a patient with extrapyramidal (Parkinsonian) rigidity.

Fig. 9. Twitch resulting from one break-shock stimulus over motor point of muscle.

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THE IMPULSE DISCHARGE FROM THE PACINIAN CORPUSCLE.

By E. D. ADRIAN and KARL UMRATH (*Graz*).

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The main object of the present work has been to study the discharge of nerve impulses from a mammalian sense organ of the encapsulated type. Adrian and Bronk(1) have recently drawn attention to the resemblance between the discharges of sense organs and of motor neurones and have suggested that both are determined by some general property of the dendritic expansion. But the most complete data for the sensory discharge have come from the tension receptors in frog's muscle. These are probably muscle spindles, but free nerve endings are usually present as well (cf. Denny-Brown(2)), and it is at least arguable that in this and in the other preparations which have been examined the discharge has always been derived from receptors of very simple type. Evidently the comparison between sensory and motor discharges would gain greatly if it were shown that a complex mammalian receptor organ is no exception to the rule.

The corpuscle of Vater and Pacini is an encapsulated organ which is so large that it can be detected easily by the naked eye. An unsuccessful attempt to record its discharge was made by Adrian and Zotterman in 1926, but the improvements in technique since then made a renewed attempt more hopeful. The only doubt arose in regard to the nature of the stimulus to which the corpuscles respond. Their distribution in the body has usually been taken to indicate that they serve as receptors for mechanical stimuli, but they are sometimes regarded as temperature organs. As will be seen the former view is undoubtedly correct.

Structure and distribution of the Pacinian corpuscles.

The corpuscles are translucent, oval bodies with a distinct white core. The largest are 3 mm. long by 2 mm. wide and the smallest about 0·2 mm. long. They are very widely distributed in the subcutaneous tissues of the fingers (in man), in the palm of the hand and sole of the

foot, the total number in the hand reaching several hundred. They are found in the neighbourhood of tendons and of joints, particularly on the flexor aspect, on the interosseous membranes and in the fascial planes in and around the muscles. Finally they are present in the abdomen in or behind the pancreas and in the neighbourhood of the solar plexus. In the cat they are scattered throughout the pancreas and are particularly numerous in the mesentery, though they occur here in many other animals.

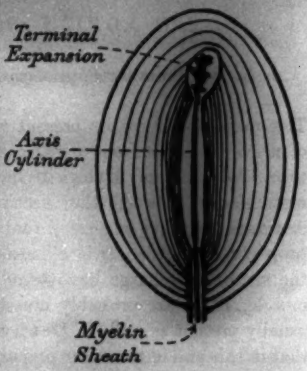

Fig. 1. Structure of Pacinian corpuscle.

Their structure has been very thoroughly worked out and the main features are summarized in the diagram in Fig. 1. The corpuscle is made of a number of concentric lamellæ of connective tissue surrounding a cylindrical core. A single medullated nerve fibre enters the central core of the corpuscle, loses its medullary sheath and passes up for some distance as a naked axis cylinder embedded in a homogeneous substance. The central core expands into a distinct chamber at the upper pole of the corpuscle, and in this chamber the axis cylinder ends in a mass of terminal arborizations. A fine non-medullated fibre is said to arborize over the outer surface of the core.

Method.

All our experiments have been made on cats decerebrated or decapitated under deep anæsthesia. The main nerve trunks of both hind limbs were cut at the hip. The corpuscles investigated were those in the hind foot. Here they are scattered in the plantar fascia, usually in close relation to the main nerve trunks and blood vessels; in the toes they occur in the subcutaneous tissue, a few are usually found at the sides of the plantar tendon sheaths and there is always a small clump of eight to ten or more corpuscles on either side of the second phalanx beneath the tendons (Fig. 2). This group is the most convenient preparation to use, since the stimuli can be applied through the tendon sheath without exposing the corpuscles, or interfering with the blood supply. The group is innervated by a fine branch of the plantar digital nerve, the main trunk of which supplies the toe pad.

For recording the responses we have abandoned the use of the capillary electrometer and installed a standard model of the Matthews

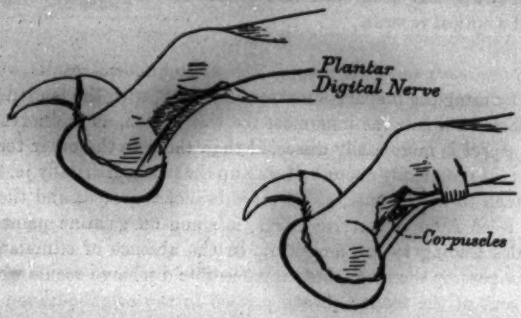

Fig. 2. Nerve supply to group of corpuscles beneath flexor tendons of cat's toe.

oscillograph(3), made by the Clifton Instrument Co., Bristol, and very kindly presented to one of us by its originator. The instrument has a period of 0·0002 second and its greatest advantage over the capillary lies in its ability to give a direct record of the exact time relations of a nerve response. In the present investigation we have not been specially concerned with this point, but the oscillograph has another outstanding merit for physiological work in that it cannot be damaged by large fluctuations of input potential. Permanent damage to a capillary tube from this cause was a rare event, but a temporary breakdown was liable to occur unless care was taken to short circuit the instrument except during the moment of recording. With the oscillograph the experimenter is relieved from all anxiety on this score, for the instrument need not be short circuited at any time throughout an experiment involving constant readjustment of electrodes, etc. Further advantages are the greater clearness of the records of irregular, high frequency discharges and the brighter image thrown on the camera slit. We have had no trouble of any kind with the instrument and have found it extremely easy to use. The amplifying system consists of the original three-valve amplifier previously used with the capillary, an intermediate amplifier of one valve with separate batteries, and an output amplifier on the lines of the standard pattern designed for the oscillograph. The photographic recording system is that previously used with the capillary, and the optical systems are arranged so that simultaneous records can be

made on the same strip of bromide paper by both instruments. The additional amplifier and loud speaker arrangement described by Adrian and Bronk(1) has been used throughout the experiments to supplement the optical records.

Results.

Sub-tendinous corpuscle group. The most uniform results were obtained by using the preparation shown in Fig. 2, with the tendon sheath left intact. As a rule the innermost toe was chosen, as the nerve on its mesial aspect is more easily dissected than those to the other toes. The nerve is cut proximally about half-way up the foot and distally just below the branch to the corpuscles. All other branches are cut and the nerve trunk, freed from connective tissue, is slung on gelatine paint brush electrodes leading to the amplifier. In the absence of stimulation no impulses pass up the nerve, but an immediate discharge occurs when the toe is bent or the tendon sheath pressed in the neighbourhood of the corpuscle group.

The number of medullated fibres in the nerve branch is of the same order as the number of corpuscles, *i.e.* about six to ten, and as one nerve fibre may supply more than one corpuscle, we cannot be sure that other kinds of end organ take no part in the discharge. Staining with methylene blue did not reveal any other kind, but the nerve fibres are difficult to trace through the mass of fat which surrounds the corpuscles. But it will be shown later that individual corpuscles react to mechanical stimulation, and there is therefore no reason to doubt that the discharges recorded from this preparation must be due mainly if not entirely to the corpuscles under the tendon.

Joint movement. Typical impulse discharges produced by bending the toe are shown in Fig. 3. The second phalanx is flexed and extended on the first by a light vulcanite rod attached to the side of the toe. As the nerves to the muscles have been cut, the tendons are slack and the discharge may differ in some respects from that occurring under natural conditions. In our experiments the total frequency of the discharge depends mainly on the rate at which the movement takes place, and a rapid movement through a small angle from the resting position produces a greater discharge than a slow movement through a much greater angle. Both flexion and extension produce a discharge of much the same character. When the toe is moved into a position of extreme flexion or extension and held there, occasional impulses continue to be discharged for a few seconds, but eventually all activity ceases.

The discharges of the individual nerve fibres can often be made out

owing to the fact that the responses fall into several groups of differing forms. Two such groups may be seen in Fig. 3 A. As one of the leads

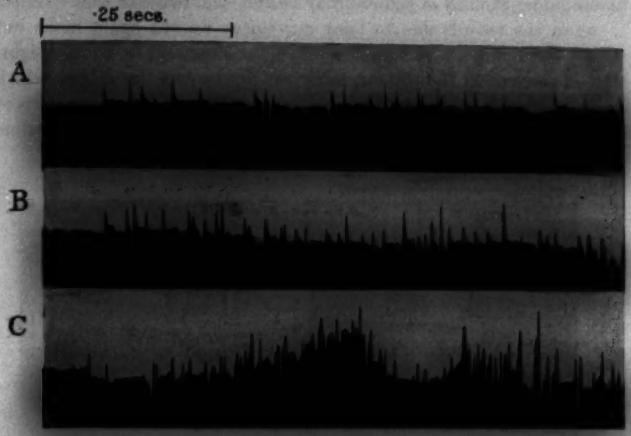

Fig. 3. Impulse discharges produced by joint movement (flexion of distal phalanx). Preparation as shown in Fig. 2 with tendon sheath intact. *A*, slow flexion; *B*, more rapid; C_1 very rapid.

was on the proximal, cut end of the nerve the variations in the amount of injury of the different fibres would account for these differences in the form of the action currents. From the records it appears that with a very slow joint movement not more than one or two nerve fibres are in action and that the discharge frequency in them may be less than 10 a second. Rhythms as high as 40 a second can be made out with very rapid movement, and the greater complexity of the discharge shows that more fibres have come into play. The total frequency may reach 250 a second for very short periods; assuming that six or seven nerve fibres are in action this gives an average discharge frequency in each of 35–40 a second.

Pressure stimuli. When the tendon sheath is pressed with the rounded end of a glass rod the point of maximum sensitivity lies immediately over the corpuscle group, though pressure elsewhere may produce an impulse discharge if it is hard enough to cause a general deformation of the sheath. When a steady pressure of 20–40 g. is maintained by a rod attached to a weighted lever the discharge usually continues for five minutes or more at a frequency which declines at first very rapidly and

then much more gradually. With small pressures (10 g. or less) the discharge may subside within a few seconds, though the yielding of the tissues under the end of the rod may be the chief cause of this.

As in the records due to joint movement we can again make out several distinct series of impulses when the discharge is at its height. Most of these seem to drop out fairly rapidly leaving one or two which maintain a moderately regular discharge over long periods. Examples of this are shown in Fig. 4, and Fig. 5 gives two curves showing the decline

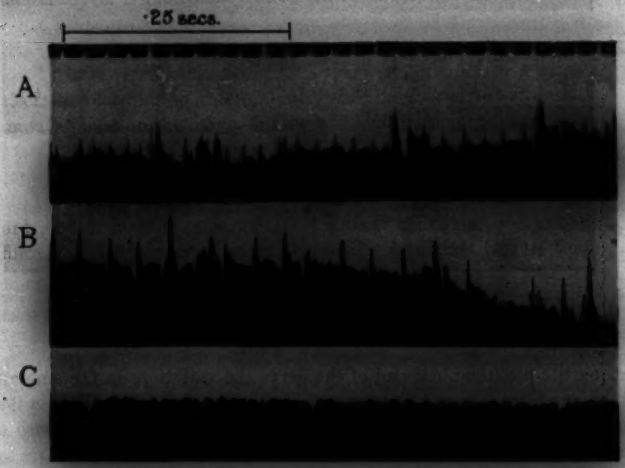

Fig. 4. Impulse discharges produced by steady pressure on tendon sheath over corpuscles. Preparation as in Fig. 2. Pressure by vulcanite rod ending in disc 11 sq. mm. area, loaded with 40 g. *A*, ½ second after beginning of loading, and *B*, 27 seconds after beginning of loading, from same record. Regular series in *A* at 51–48 a second; in *B* at 31–29 a second. *C*, another record, pressure at a point to one side of most sensitive region, 7 seconds after beginning of discharge, frequency about 12 a second.

in frequency of a regular series of this kind. These curves differ very little in form from those constructed by Adrian and Zotterman (4) for pressure applied to the cat's toe pad, though in the latter the nature of the receptor was uncertain and the curves refer to the total frequency of the discharge and not to that of a single series of impulses.

Temperature stimuli. It is fairly clear that the discharges recorded in this way are derived from the Pacinian corpuscles and that they serve

to convey information of the movements of the toe joint and possibly also of the amount of pull exerted by the tendon. It was therefore unlikely that temperature stimuli would produce any discharge when

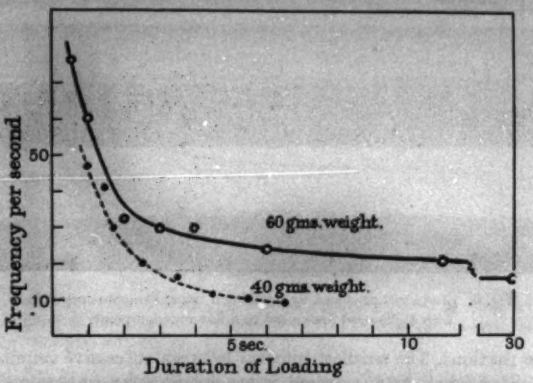

Fig. 5. Decline in frequency of a regular series of impulses produced by continued pressure over tendon sheath with disc 11 sq. mm. in area.

applied over the region of the corpuscles. The temperature of the preparation was controlled by irrigation with warm Ringer and by warm pads, and it was noticeable that no discharge occurred on irrigation with Ringer at different temperatures provided that mechanical stimulation was avoided. To test the point more thoroughly, water at different temperatures was passed through a metal tube held by a clamp in contact with the tendon sheath. The results were entirely negative although the rise and fall of temperature covered a wide range and occurred quite rapidly enough to be detected by a finger resting on the tube. On the other hand, if the cutaneous branches of the nerve were left uncut and the tube was placed at the side of the toe pad, the change from hot to cold gave a definite impulse discharge.

Stimulation of single corpuscles. Pacinian corpuscles are often found in groups of two or three on the surface of the tendon sheath, and we found it possible to stimulate a single corpuscle by pressing on it by hand with a glass rod drawn out to a small rounded point or by a fairly stiff hair. A record of the discharge produced in this way is shown in Fig. 6. The impulses tend to occur in small groups, separated by occasional impulses less regularly spaced. The fluctuations of pressure are

probably enough to explain this type of discharge though further experiments may be advisable to account for the sudden appearance of such

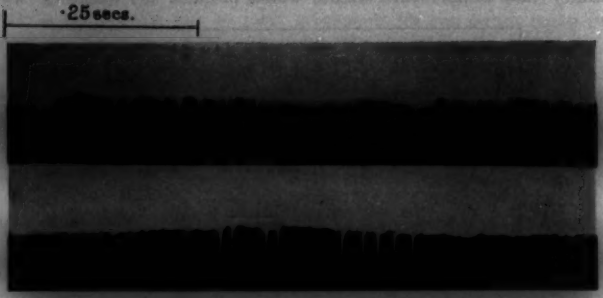

Fig. 6. Discharges produced by touching a single Pacinian corpuscle with a glass rod drawn out to a fine rounded point.

definite rhythms. The smallest interval between successive impulses in our records has been 1/160 a second, but such intervals were obtained with stimuli which produced considerable deformation of the corpuscle and were probably outside the range of those to which it would be normally subjected.

We were not able to determine the effect of steady pressure on a single corpuscle, but a weighted lever resting on a group of two or three corpuscles gives a discharge like that obtained by pressure over the tendon sheath, with one or more fairly regular rhythms declining gradually in frequency. A curve illustrating this is given in Fig. 7, and it will be seen that it agrees closely with the curves in Fig. 5.

Many attempts have been made to record impulses from the corpuscles which occur singly along the more proximal part of the nerve trunk where it is bound down by the plantar fascia, but difficulties arise from the presence of other sensory endings in the fascia or blood vessels. In some preparations these may give rise to spontaneous discharges which persist as long as the nerve fibres to the fascia are intact, in others a discharge is produced whenever the fascia is stretched. Such discharges occur after the destruction of all the Pacinian corpuscles visible with a dissecting microscope, and their occurrence raises the question whether all the discharges recorded in our experiments may not have come from endings near to but not actually in the corpuscles. In preparations from the toe, however, where the thick fascia is absent, the

likelihood of this must be very small. No discharge can be obtained by movement of the surrounding tissues after the corpuscle groups have

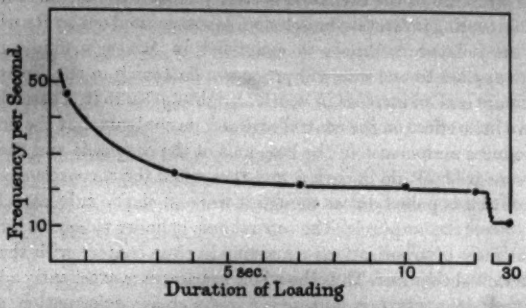

Fig. 7. Decline in frequency of a regular series of impulses produced by continued pressure on a group of three exposed corpuscles. Pressure by disc 11 sq. mm. area, loaded with 40 g. Compare with Fig. 5.

been destroyed, and although pressure with a pointed rod in the neighbourhood of a corpuscle may give rise to a few impulses, the discharge is much greater when the corpuscle itself is touched.

The functions and mechanism of the corpuscle. That the Pacinian corpuscle is an organ responding to pressure stimuli is a view which has been widely held on the basis of its structure and its distribution in the body (cf. Sherrington(5)). Our results confirm this view, and in so doing they emphasize the importance of the corpuscle in regard to sensations from the joints and from the viscera. Sensations of joint movement are probably derived from more than one type of end organ. In the frog, which has no Pacinian corpuscles, we have found that an impulse discharge in the sciatic nerve is usually produced by movement of the knee joint after all nerve branches to the muscles have been cut, though in some preparations the discharge has only appeared with an extreme degree of movement which would be likely to stimulate pain endings. In mammals the corpuscles which are in close relation to the joints will evidently signal the changes of pressure produced by the joint movement, though other forms of ending may take some part in the message. Von Frey(6) has shown that the extent of a finger movement in man can be appreciated after the sensory nerves to the finger have been put out of action, and he considers that in these circumstances the Pacinian corpuscles in the tendon sheaths supply the necessary

information, although they may be some distance from the joint itself.

The structure of the corpuscle is clearly related to its function as an organ for reacting to lateral compression, as was pointed out by Rauber(7). When an isolated corpuscle is examined in Ringer's fluid, a light pressure applied to one side will produce a flattening in the direction of the pressure and an increase in width at right angles to it. These changes can have little effect on the central core and its contents, but the pressure also produces an increase in the long axis of the corpuscle and the walls of the core are built up in such a way that when the corpuscle elongates the core itself is pulled out as though it were an elastic tube attached to either pole of the corpuscle. The central axis cylinder elongates with the core, the mass of arborizations remaining in close contact with the walls of the terminal chamber. Thus the whole apparatus is excellently adapted for producing a varying, but never very great, deformation of the delicate nerve terminations in response to varying pressure.

From this point of view the function of the capsule appears to be purely mechanical and its action is comparable to that of a system of levers working against an elastic restraint. It will react to a pressure in almost any direction and the thickness of its walls will determine the range of pressure needed to produce a given deformation of the nerve endings.

We have no reliable data as to the range of pressures which are normally effective, for the exposed corpuscles are liable to give inconstant results. In some preparations a hair which bends at 20 mg. has produced a discharge when moved to and fro on a group of corpuscles. On the other hand pressure with a disc 11 sq. mm. in area and loaded with 60 g. is not great enough to cause an obvious loss of responsiveness, though the yielding of the soft tissue under the corpuscles may protect them from the full effect of such pressure. According to Rauber pressures of a few milligrammes are quite enough to produce some elongation of the corpuscle and the maximum elongation is reached with pressure of 1–2 g. It has been suggested (Schumacher(8)) that the corpuscles may respond to changes of blood-pressure owing either to the increased diameter of the vessels near them or to the altered distribution of fluid in or around them. It is possible that these changes may sometimes provide an adequate stimulus, but adequate stimuli due to movement and external pressure must occur so often that it is unlikely that the corpuscle can have any specific function in connection with blood-pressure or fluid regulation.

Discussion.

It is satisfactory to find that the impulse discharge from a complex mammalian sense organ has the same general features as those already observed in various sensory discharges. The frequency depends on the intensity of the stimulus; under continued stimulation the impulses form a regular series with a gradually decreasing frequency, and for stimuli of normal intensity the frequency in each nerve fibre usually falls within a range of about 60–10 a second. In all these points the discharge agrees closely with that of the tension receptors in the frog's muscle; in the frog's skin (where the receptors are free nerve endings) the only significant difference lies in the more rapid decline of the discharge. Thus the statement that receptors of varying structure show no characteristic differences in discharge frequency is no longer open to doubt on the ground that histological evidence of varying structure was lacking.

The result is of importance because it confirms the remarkable likeness between the discharge of the sense organs and of the motor neurones to skeletal muscle. Here, too, the frequencies commonly encountered range between 10 and 60 a second, the frequency varies with the intensity of the excitation and the discharge has the same rhythmic character, although the intervals between successive impulses show a slightly greater fluctuation.

The general resemblance of the discharges may be insisted on without implying that the entire range of frequency is the same in every case. There is a common lower limit of about 5 a second in the sense that discharges of lower frequency are usually irregular, but the upper limit is much less definite. With very intense stimulation Matthews has shown that the tension receptor in frog's muscle may respond as rapidly as 250 a second or more, whereas in the motor fibres of the cat Adrian and Bronk did not find frequencies above 100 a second. When the whole innervation, sensory and motor, of a muscle is intact, the maximum frequency of the motor discharge may be reflexly adjusted to suit the type of muscle fibre. Thus, in the muscle fibres of the soleus (red) in the decerebrate cat we have not found rhythms higher than 34 a second, whereas the gastrocnemius gives rhythms of 50–60 a second. This agrees with Denny-Brown's findings (9), and he has pointed out that the lower values from the soleus are probably due to an inhibitory restraint from receptors in the muscle itself since the frequency may be higher in the

deafferented muscle[1]. Nevertheless it remains a striking fact that a discharge of impulses at a frequency of, say, 30 a second might be due to the normal activity of such unlike structures as the motor nerve cell, the Pacinian corpuscle and the receptors in the frog's skin and muscle. One feature common to all of them is the possession of a medullated axon, and as the frequency is actually measured in the nerve and not in the sense organ or nerve cell it is worth while enquiring whether it may not be determined by some property of the medullated fibre itself. An explanation based on the gradual return of excitability in the nerve fibre was rejected by Adrian and Zotterman(10) on the ground that it would only account for much higher frequencies, but it is true that there are other recovery phenomena which proceed much more slowly than the return of excitability and there are conditions under which this also is prolonged. At present, however, it cannot be said that any of the known properties of the medullated fibre provide an obvious basis for discharge frequencies as low as 30 a second. The simplest alternative is to suppose that the rhythmic discharge actually starts in the terminal arborizations of the sense organ and in some part of the motor nerve cell or its dendrites, and that the common factor is to be found in the nature of their exposed surfaces. If the discharge is due to the repeated breakdown and rebuilding of a surface film, its character may depend entirely on the nature of this film without regard to the particular structure in which it occurs or to the particular type of stimulus which disturbs the surface equilibrium. A slightly different hypothesis would be that the rhythmic process originates at the junction of the medullated and non-medullated part of the axon; this would make it possible to account for differences in frequency range corresponding to differences in the character and size of the sheath, but as these have yet to be detected, there is little to choose at the moment between this and the former view.

Whatever the common factor may be it has to explain the fact that different forms of stimulus acting on different structures all produce a discharge of the same kind. For instance, the motor neurone stimulated by the impulse streams which converge on it from other neurones gives a discharge indistinguishable from that of a sense organ responding to mechanical deformation. It is possible that the stimulus which actually takes effect on the nervous substance is always the same, for it may be

[1] Preliminary observations on the motor discharge in the tortoise indicate a very low range of frequency, but we have not yet enough evidence to say how far this is dependent on inhibition from the muscles.

something which is liberated in the region of the sensory ending or of the motor neurone. In any case the particular structure must determine what kind of change becomes an effective stimulus under normal conditions, but it is of interest to enquire whether the same kind of discharge may not be produced by abnormal stimulation as well, *i.e.* whether a motor neurone will not react in the same way to mechanical as well as to reflex disturbance. It is well known that pressure on the exposed spinal cord may produce vigorous and sustained contractions. The reaction must be due to the grey matter, for compression of the dorsal roots gives no more than a brief twitch. If it is true that the rhythmic discharge, varying in frequency over a given range, is the normal response of certain forms of nervous substance, however stimulated, we ought to find that mechanical stimulation of the cord gives a discharge in the motor nerve fibres of the same type as that produced in sensory fibres by mechanical stimulation of the end organs.

Mechanical stimulation of the spinal cord.

Records of the electromyogram obtained by touching the spinal cord and leading off from the contracting muscles to a string galvanometer have been published by Dusser de Barenne and Brevée(11) in 1926. With the cord intact they found an electromyogram made up of rapid irregular oscillations of high frequency. The oscillations resembled those found in the electromyogram of voluntary or reflex contractions, but as the records were made from leads which would include the greater part of the muscle, the frequency of discharge of the individual neurones was uncertain. They obtained more definite results by treating the dorsal surface of the cord with novocaine solution. This caused a reduction in the size of the contractions, but the waves in the electromyogram became slower and more regular and eventually reached a definite rhythm at 50 a second. They considered that the novocaine produced this simplification of the response by putting the greater part of the nervous mechanism out of action, leaving the inherent rhythm of the motor neurones to assert itself. In the light of our present knowledge of the electromyogram it seems that the appearance of a definite rhythm must be due mainly to a greater degree of synchronous activity in the neurones, though it is not clear why this should occur. Thus Dusser de Barenne and Brevée results have shown that mechanical stimulation of the cord may give a motor neurone discharge with a rhythm of 50 a second, but it is uncertain how far this rhythm is due to the action of the novocaine.

Several methods are now available for determining the frequency of discharge in individual motor neurones. The simplest consists in leading off from the contracting muscle with a very small electrode system. Adrian and Bronk used two forms of electrode consisting of one or two wires sheathed in a hypodermic needle, and with either system the distance between the leads is so small that the action currents are mainly derived from the few muscle fibres in contact with the needle

point. We have used this method to determine the frequency when the cord is stimulated mechanically, and the results are quite definite.

The experiments were made on decapitated cats. The spinal cord was exposed in the cervical region and stimulated by touching its dorsal surface with the rounded end of a glass rod. The response to each touch may be no more than a sudden twitch of the foreleg, but as Dusser de Barenne and Brevée observed, repeated touches every second often produce a sustained contraction of the biceps or triceps, and a firm pressure momentarily applied may give a contraction persisting for several seconds. The needle electrode system was thrust into the muscle which seemed to contract most vigorously, and the amplified action currents were examined in the usual way by photographic recording and conversion into sound waves. Portions of two records are given in Fig. 8. The upper shows a rhythm of 26 a second, and in the lower the

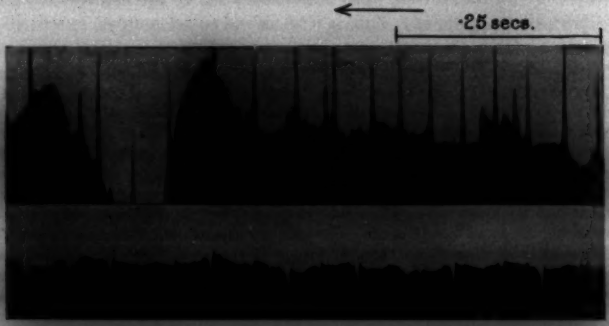

Fig. 8. Electric responses of biceps during contraction produced by pressure on exposed spinal cord (decapitate preparation). Concentric needle electrodes. Compare with Fig. 4. (N.B. These records read from right to left.)

frequency of the main series is 9 a second. These and many other records show that regular discharges may occur with frequencies much lower than 50 a second and without the use of novocaine. As in a reflex the frequency decreases gradually when the contraction declines. It may increase gradually at the outset, but more often there is an abrupt and powerful contraction at the moment when the cord is touched, and it is usually impossible to distinguish the rhythms of individual fibre groups until a steady contraction supervenes. The maximum frequency is therefore uncertain, but during the sustained contraction the records

are indistinguishable from those obtained from muscles in reflex or voluntary activity, and a comparison of Fig. 4 shows that they can only be distinguished from a sensory nerve fibre discharge by the different form of the potential waves.

Thus mechanical stimulation of the grey matter produces a motor neurone response of the familiar type, and indeed the cord behaves exactly like a mass of sensory end organs connected directly with the motor fibres. Unfortunately, we cannot draw what would be a much more significant conclusion, namely, that the discharge is produced by mechanical stimulation of the motor neurones themselves. The difficulty arises from the fact that the excitation may be started on the afferent side and that any bombardment by afferent impulses is likely to produce the familiar motor neurone discharge. On the other hand Dusser de Barenne's results showed a rhythmic discharge appearing in the whole muscle at a stage when the dorsal part of the grey matter was put out of action with novocaine, and our own results make it extremely unlikely that the novocaine had changed the character of the discharge from the individual neurones.

For the present, then, these results are suggestive but not conclusive, and the most that can be said is that they do not reveal any other kind of response when the grey matter is subjected to an unusual form of stimulation. To this extent they support the view that there is some fundamental property in the sensory endings and the neurones of the cord which must account for the general likeness between sensory and motor discharges.

Summary.

The Pacinian corpuscles are encapsulated end organs visible to the naked eye, and their mode of action can be determined by recording the impulse discharges in the afferent nerve fibre when various forms of stimulation are applied to the corpuscle. Suitable preparations were found in the group of corpuscles under the flexor tendons of the cat's toe and in the scattered corpuscles on the surface of the tendon sheath. The nerve impulses were recorded by an amplifier system in conjunction with a Matthews oscillograph.

The following conclusions are drawn:

1. Changes of temperature do not excite, but pressure gives an immediate discharge of impulses from the corpuscle. Moving the phalangeal joint is an effective stimulus to the corpuscles below the tendon in this region, and pressure over the tendon is equally effective. A single corpuscle can be stimulated by pressing it with a glass rod or a hair.

2. The discharge is of the same type as that from the receptors in the frog's muscle and skin and in the cat's toe pad. The frequency varies with the pressure and declines gradually under a constant stimulus. A single corpuscle gives a rhythmic discharge with a frequency which varies from about 5 to about 100 a second for stimuli of normal intensity.

3. The discharge of a single mammalian sense organ is of the same type, and for normal stimulation it covers approximately the same range of frequency as that of the sense organs in the frog and of the motor neurones of the spinal cord excited reflexly and voluntarily. It is suggested that the character of the discharge is determined by some property common to all neurones which have a medullated axon, the reaction being the same whatever the nature of the stimulus.

4. In support of this it is found that mechanical stimulation of the exposed spinal cord produces a motor neurone discharge which is indistinguishable from that due to reflex activity and from that produced in a sensory fibre by mechanical stimulation of its nerve ending.

The expenses of this research were defrayed by a grant from the Foulerton Fund Committee of the Royal Society, and we are greatly indebted to Mr B. C. Matthews for his valuable gift of the oscillograph with which the records were made.

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THE PHYSICO-CHEMICAL PROPERTIES OF THE VITREOUS BODY.

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In a previous series of papers(1) experimental evidence was brought forward that the aqueous humour was a dialysate of the capillary blood, and, in an examination of the consequences of this theory on the intra-ocular pressure, long "lag" effects were noted in variations of the pressure which seemed to be most easily explained by physico-chemical changes occurring in the vitreous body. The present studies were undertaken with a view to elucidate these.

The vitreous body has always presented difficulties in its examination, owing to its transparency and the great delicacy of its structure. It is generally described histologically as being made up of a frame-work of fibres forming a net-work enclosing a fluid in its meshes, but the fact that great variations appear with the different fixatives employed and with the state of preservation of the specimen, has at various times given rise to the suspicion that these appearances might be artifacts of an extremely misleading nature. In view of this it would seem desirable that a more unequivocal means of investigation be employed; and for the study of a transparent mass of this kind the ultra-microscope seems to be the most suitable, since it necessitates neither fixatives nor any procedures such as would tend to introduce artifacts, but allows the fresh and unaltered material to be studied directly. It was first employed for the study of the vitreous body by Baurmann (1923(2)), whose paper was followed by the observations of Heesch (1926(3)), all of whose conclusions I have confirmed.

Ultra-microscopic examination of the vitreous body demonstrates that it is a true gel without any microscopical structure in the usual sense of the word. At first the fresh vitreous is optically empty, but very soon innumerable minute fibrillæ appear in the beam of light, 3 μ long by 2 μ thick, criss-crossing each other in all directions (Fig. 1). After standing for an hour these become restless and begin to flit about from place to place; the fibrillæ appear to break down into rows of separate particles resembling strings of beads, until finally only separate dots are seen (Fig. 2). These appearances resemble very closely those described by

Zsigmondy and Bachmann(4) in the ultra-microscopic examination of soap gels.

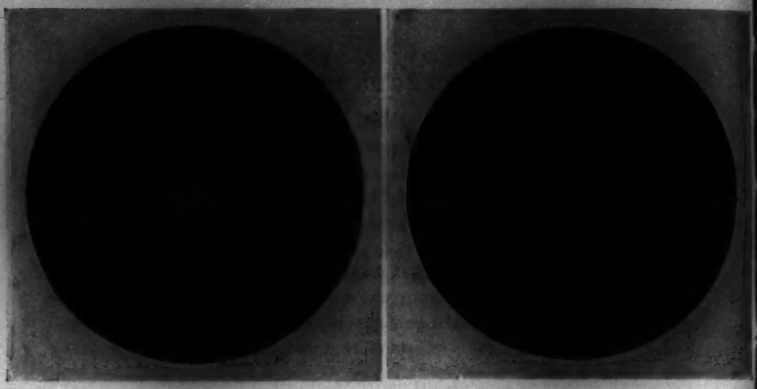

Fig. 1. Fig. 2

Fig. 1. Six hours old vitreous seen by ultra-microscope.

Fig. 2. Twenty-four hours old vitreous seen by ultra-microscope.

The "fibres" of microscopic size seen in histological preparations seem to be merely the coagulation of these micellæ by the fixative employed: and the membranous appearances seen clinically with the slit-lamp appear to be due to the orientation of the micellæ in approximately parallel directions and in closer formations near the surface boundary layers, while in regions where the arrangement is more haphazard the vitreous appears optically empty. This apparent optical structure is complicated by the fact that there are inter-faces within the vitreous body; for the part immediately behind the lens and occupying the canal of Cloquet (which has a separate and more primitive embryological origin) is of looser structure and is differentiated from the remainder by a distinct surface layer. A similar surface condensation appears round the main mass of the vitreous and gives rise to the appearance of the "hyaloid membrane," the separate existence of which as an actual membrane has been disputed for nearly half a century.

If the fresh vitreous is suspended on a clamp, or if it is filtered through an ordinary filter paper, the gel structure gradually breaks down and drops of fluid appear, until finally the whole of the mass is collected as a clear solution of a soapy consistence, leaving hanging to the clamp or stretched out upon the filter paper a thin film of homogeneous material. This simple experiment has been frequently done before, most recently by Redslob(5), and the residue has been described as the fibrous scaffolding of the vitreous (an explanation which takes no account of

the complete homogeneity of the material), or, alternatively, as the hyaloid membrane (an explanation which neglects the fact that the residue may be obtained in identical form from any portion of the vitreous body utilized, peripheral or central). It seems that this residue is a structureless and homogeneous material which is uniformly distributed throughout the mass of gel. Its separation with the liquefaction of the whole structure of the gel is not due to bacterial or fermentative activity, for the same phenomenon occurs, little changed in its time relations, if the process is carried out at $-7°$ C. There is no similar formation after prolonged centrifugalization. The experiment seems to be a demonstration that the vitreous is a gel in an unstable state of dynamic equilibrium, the consistence of which can be readily destroyed by mechanical trauma.

Chemical composition.

The chemical composition of the vitreous body has never been completely determined by any investigator, although many have made partial analyses involving several of its constituents, more especially the mineral salts. The most recent work is that of Cohen, Killian and Metzgar(6), and Tron(7). It would seem advisable that such an analysis should be undertaken with a view to elucidating the nature and properties of the gel.

This was done on the vitreous of horses: over 1000 eyes, collected from abattoirs immediately after slaughter, were employed, so that the material may be taken as a fair average sample. The results of the analysis were given in the accompanying table, where they are compared with an analysis of the aqueous humour conducted throughout on the same lines, and also with an analysis of a typical sample of horse serum taken from the farm of the Medical Research Council(8).

The total protein was estimated gravimetrically by coagulating by heat. A large quantity of whole vitreous was now filtered and the residue was similarly washed, dried, and weighed; this is called "residual protein" in the table. The filtrate was acidified by acetic acid, when the copious precipitate of muco-protein was estimated gravimetrically. This second filtrate was used in all subsequent analyses and is referred to as filtrate *X*. The total proteins still remaining in it (albumin and globulin) were estimated gravimetrically by heat coagulation. In a second portion of the filtrate which had been neutralized and concentrated, the albumin was determined gravimetrically by precipitating the globulins by ammonium sulphate, a fractional precipitation being repeated five times. The globulin was taken as the difference between this value and that of

The chemical composition of the vitreous body compared with that of the aqueous humour and the blood serum.

(*Horse.*)

Quantities in g. per 100 c.c.	Vitreous	Aqueous	Serum
Water	99·6813	99·6921	93·3238
Solids (dried at 100° C.)	1·1087	1·0869	9·5362
Total protein	0·0652	0·0201	7·3692
Albumin	0·0077	0·0078	2·9557
Globulin	0·0115	0·0123	4·4135
Muco-protein	0·0211	—	—
Residual protein	0·0250	—	—
Fibrinogen	Traces	Traces	Present
Immune bodies	Traces	Traces	Present
Ferments	Traces	Traces	Present
Fats	0·007	0·004	0·13
Cholesterol	0·0005	Traces?	Present
Non-protein nitrogen	—	—	0·0239
Total nitrogen	0·0301	0·0268	—
Urea	0·029	0·028	0·027
Amino-acids	0·030	0·029	0·035
Lactic acid	0·019	0·205	Present
Creatinine	0·001	0·002	0·002
Sugar	0·0973	0·0983	0·0910
Sodium	0·2731	0·2787	0·3351
Potassium	0·0192	0·0189	0·0201
Calcium	0·0068	0·0062	0·0101
Magnesium	0·0020	0·0026	0·0028
Chlorine	0·4168	0·4371	0·3664
Phosphorus (inorganic)	0·0031	0·0033	0·0030
Sulphur (inorganic)	0·0062	0·0061	0·0058

the total albumin and globulin. The total nitrogen of the vitreous body was estimated by a micro-Kjeldahl determination; urea was estimated in filtrate *X* by the urease method; the amino-acids by Van Slyke's method; and the creatinine colorimetrically against a standard creatinine picrate solution.

By the term "fats" is meant the figure obtained by weighing an ether extract of dried residue. The cholesterol was estimated after extraction of filtrate *X* by precipitation with digitonin. "Sugar" is taken as reducing substance estimated in filtrate *X* as glucose by the Schaffer-Hartmann method.

The total mineral ash was obtained by calcination. The material thus left was dissolved in HCl; barium chloride and baryta were added, and the phosphates and sulphates removed; barium salts were removed by ammonia and ammonium carbonate; the filtrate was evaporated to dryness and the ammonium salts removed by heat; the residue was dissolved, treated again with ammonia and ammonium carbonate,

filtered, acidified with HCl and evaporated to dryness. This residue was redissolved; the potassium was estimated by precipitation with H_2PtCl_6, and the amount of sodium calculated by difference. The calcium was precipitated as oxalate, and estimated as CaO, the magnesium as ammonium magnesium phosphate and estimated as magnesium pyrophosphate. The chlorides were determined by Ruszynák's modification of Koranyi's method; the inorganic phosphates gravimetrically as pyrophosphate; and the inorganic sulphates by conversion to benzidine sulphate and titration with sodium hydroxide.

From the table of analysis it is evident that all the constituents of the aqueous humour are present in the vitreous body, and that, within the limits of experimental error, they are present in comparable proportions. The only substances which are present in the vitreous body and are not present in the aqueous humour are the muco-protein and the material which has been provisionally termed "residual protein."

The muco-protein of the vitreous body. The muco-protein of the vitreous body has been investigated by Levene(9), who established it to be a compound of mucoitin sulphuric acid *B*, and to contain chitosamine as a carbohydrate radicle. It liberates a reducing sugar after hydrolysis. It is most readily precipitated at *p*H 2·5. A similar substance occurs in the cornea and the Wharton's jelly of the umbilical cord; and it is noteworthy that its presence in all three cases is associated with optical transparency.

The residual protein. The material which is separated from the vitreous gel by filtration is extremely difficult to investigate chemically, owing to its inert nature and to the very minute quantities in which it can be obtained, even after the expenditure of an enormous mass of material. With the material at our disposal we have not been able to decide definitely whether it is a single substance or not. It appears as a sticky, gummy material, which, after washing and drying, resembles a dry and horny piece of gelatin. It does not dissolve in water, but is extremely hygroscopic; it swells up into a clear gel, soluble to a slight degree in acids, and to a very marked degree in alkalis, and it completely and readily dissolves on boiling with either acids or alkalis. Its elementary composition is as follows:

	p.c.
C	44·64
H	6·415
N	12·20
Total ash	3·825
P	0·12
S	0·675

This composition suggests a protein substance allied to the muco-proteins. The fact, however, that it gives no reducing action after hydrolysis and that no carbohydrate radicles (either hexoses or pentoses) can be detected seems to negative this[1]. It is not digested by pepsin or trypsin, and gives a negative result to all the usual gross tests for protein. On the whole, considering its inert nature, the absence of carbohydrate, its ability to withstand the action of ferments, and its (presumably) ectodermal origin from the neuroglial elements of the retina, it would seem most probable that it is related to the group of kerato-proteins[2], although its small sulphur content seems to negative a close association.

The importance of this substance appears to be its power of forming a gel. If the proteins of the whole vitreous body are coagulated by treatment with alcohol and extraction with ether at — 5° C., a mass of gel can be obtained (an artificial vitreous) by the simple addition of water; if, however, the proteins of a filtrate of the vitreous (that is, in the absence of this constituent) are similarly treated, gel formation seems impossible. The extreme dilution in which this substance is capable of gel formation *in vitro* is especially remarkable (0·025 p.c.); and it would seem that, like the muco-protein, it has a very special function.

As the result of these chemical studies, we conclude that the vitreous is formed of two substances peculiar to itself, each with a special function (the maintenance of transparency, and the potentiality of gelability) plus the constituents of the aqueous humour in comparable proportions. The thesis has been recently advocated that the aqueous humour is a dialysate from the capillary blood, the dialysing membrane being the capillary walls (1). So far as the chemical part of the argument was concerned this theory was based upon the fact that the aqueous humour was found to contain traces of the serum proteins, while the non-dissociated diffusible substances are partitioned between it and the capillary blood in practically equal concentrations when expressed as g. in an equal weight of water, and the dissociated diffusible constituents show the increase in the concentration of anions and the diminution of cations required by the thermodynamical laws related to two fluids in the membrane equilibrium described by Donnan. Recent embryological work (Mann (11)) has shown that the vitreous body appears to be

[1] For confirmation of this I am indebted to Mr John Pryde.

[2] Mörner (1893 (10)) reported that, on heating this substance to 105° C. in distilled water over a paraffin bath for five hours and then cooling, it forms a clear jelly. He called it "gluten." This, after several attempts, I have been completely unable to verify.

formed from the ectodermal elements surrounding the vitreous chamber (lens' plate and retina); it seems probable that these two constituents, the chemical nature of which suggests in each case an ectodermal origin, are thus specially secreted. That portion of the intra-ocular fluid which dialyses into the anterior chamber remains as the aqueous humour, that part which reaches the vitreous chamber, entering into physical combination with the proteins secreted here, forms a gel with these as basis.

The plasmoid vitreous body.

If these views are correct it follows that if the permeability of the membrane between the plasma and the vitreous body is altered, that the composition of the vitreous should change in the terms of a membrane equilibrium. This was shown to be the case with the "plasmoid" aqueous humour obtained after the intra-ocular capillaries were dilated(8). Experiments were done with a view to ascertain if the same phenomenon occurred in the vitreous body.

The experiments were done on rabbits, and a dilatation of the capillary walls was brought about by lowering the intra-ocular pressure suddenly by a paracentesis in which 0·25 c.c. of vitreous was abstracted. The chemical constitution of the normal vitreous body was obtained by an examination of the vitreous of one (untouched) eye of the animal; while the constitution of the abnormal vitreous was determined in the other eye at various times after paracentesis.

The chemical compositions of the normal and plasmoid vitreous bodies were compared in respect of their content of colloids and dissociated and non-dissociated diffusible constituents: the estimations were made on the filtrate of the whole vitreous body. The protein constituents were estimated by a dipping refractometer (Zeiss) fitted with an auxiliary prism to allow a single drop of fluid to be examined at a time, the increase of refractive index obtained after paracentesis being roughly translated into concentrations of protein (albumin and globumin) by the method suggested by Robertson(12). Sugar was taken as a representative of the non-dissociated diffusible substances, and its estimation was done by the micro-method of Hagedorn-Jensen. In a similar manner chloride was taken as representing a typical negatively charged diffusible constituent, the estimation being performed by Ruszynák's micro-modification of Koranyi's method. The results are expressed in the accompanying tables, and are illustrated in Fig. 3.

Proteins of vitreous body.

Time after paracentesis: 0·25 c.c. removed	N_D normal vitreous filtrate	N_D plasmoid vitreous filtrate	Difference (plasmoid – normal)	Approximate percentage proteins
1 hour	1·334935	1·336898	0·001963	1·1
	1·334818	1·335783	0·000965	0·5
	1·334896	1·335900	0·001004	0·6
			Mean	0·73
2 hours	1·334506	1·339416	0·004910	2·6
	1·334740	1·338656	0·003916	2·0
	1·334818	1·339416	0·004598	2·5
			Mean	2·37
6 hours	1·334662	1·337430	0·002768	1·5
	1·334740	1·337849	0·003109	1·7
	1·334584	1·336822	0·002238	1·2
			Mean	1·47
48 hours	1·334740	1·336436	0·001698	0·9
	1·334779	1·336592	0·001813	1·0
	1·334584	1·335861	0·001316	0·75
			Mean	0·88
6 days	1·334506	1·334701	0·000195	0·1
	1·334896	1·335168	0·000272	0·15
			Mean	0·125

Chloride of vitreous body.

Time after paracentesis: 0·25 c.c.	Normal vitreous filtrate	Plasmoid vitreous filtrate	Difference (normal – plasmoid)
1 hour	0·595	0·545	0·050
2 hours	0·650	0·532	0·118
6 hours	0·521	0·418	0·103
48 hours	0·563	0·494	0·069
6 days	0·622	0·582	0·040

Sugar of vitreous body.

Time after paracentesis: 0·25 c.c. removed	Normal vitreous	Plasmoid vitreous
2 hours	0·157	0·160
6 hours	0·136	0·138
8 hours	0·125	0·125

Aqueous humour.

Time after paracentesis: 0·25 c.c. removed	N_D normal aqueous humour	N_D plasmoid aqueous humour	Difference (plasmoid – normal)	Approximate percentage proteins
20 min.	1·335168	1·339036	0·003868	2·0
1 hour	1·335242	1·339834	0·004592	2·5
2 hours	1·335130	1·338618	0·003488	1·9
6 hours	1·335168	1·337392	0·002224	1·2
6 days	1·335244	1·335244	0·000000	0

It is seen that in the rabbit the curve tracing the variation in the protein content rises sharply to attain a maximum about two hours

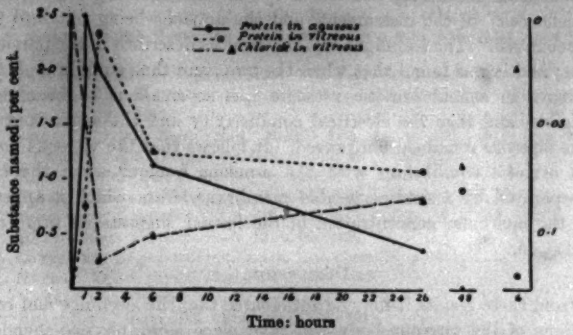

Fig. 3. Percentage of protein and chloride, in aqueous or vitreous, as a function of the time after paracentesis.

after paracentesis and then falls; after eight hours it is again approaching the normal. Ophthalmoscopic observations confirm this, for a definite turbidity in the vitreous is seen two hours after puncture, the greater part of which rapidly clears up, but of which traces can be seen for some days. The curve showing the variation in the chloride (a negatively charged ion) shows an inverse variation to that of the colloids; while the concentration of sugar (non-ionized) remains practically unchanged, any small change which does occur being most probably due to emotional excitement on the part of the animal. The changes are comparable to those noted in the composition of the aqueous humour after paracentesis, but it is interesting that they do not exactly coincide. The changes in the vitreous body take a longer time to attain a maximum, and a much longer time to resolve again to the normal, a difference which is only to be expected, inasmuch as the processes of diffusion in a gel must be considerably slower and show more inertia than the corresponding processes occurring in a sol.

The osmotic pressure of the vitreous body.

In a previous paper (13) it was concluded that so far as its molecular concentration is concerned, the aqueous humour corresponds to a dialysate of the capillary blood. The same micro-osmometer which was used

in this series of experiments was used to compare the molecular concentration of the vitreous body with that of the aqueous humour, the vitreous from the freshly enucleated eye of a rabbit being put into the "plasma cell" of the instrument, and the aqueous being put into the "aqueous cell." The technique was the same as described in the previous paper; and it was found that when the two were thus equilibrated, the difference in colloid osmotic pressure was so small as to be almost negligible, and that the electrical conductivity and the sugar content of the aqueous remained unchanged. It follows that the vitreous body is in osmotic equilibrium with the aqueous humour when the two are separated by a semi-permeable membrane, from which it appears that the molecular concentration of the former suggests its origin by dialysation.

Discussion.

From these results it may be anticipated that the swellings and contractions of the vitreous body will take place along physico-chemical lines, according to the conditions which govern the processes of turgescence and deturgescence in gels. A state of turgescence will tend to bring about a condition of hypertension in the eye; while a state of deturgescence or of liquefaction of the gel will tend to bring about a condition of hypotension. Inasmuch as it is not a tissue but a cell-product, it may also be anticipated that the reaction of the vitreous body *in vivo* to metabolic or toxic disturbances will be passive; and that, owing to the instability of the gel, one of the first results that we should expect from such disturbances will be its liquefaction with the separation of part of its colloid basis (the residual protein) to form appearances evident clinically as opacities.

Summary.

1. The physical properties of the vitreous body show that it is a simple gel.

2. Its chemical constitution in the normal condition and in the abnormal condition after paracentesis, and its osmotic concentration suggest that it is formed upon the basis of two (or more) special protein constituents elaborated by the surrounding ectoderm, and that the common intra-ocular fluid (which is a dialysate of the capillary blood) percolates these to form with them a gel.

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612.492.8:612.17.019.89 (Rabbit)

THE ACTION OF POSTERIOR PITUITARY PRESSOR EXTRACT ON THE RABBIT'S VASCULAR SYSTEM.

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In the course of experiments to compare the effects of posterior pituitary extract on the blood sugar of well-fed rabbits with those in rabbits whose glycogen reserves had been depleted by thyroid feeding, it was found that animals which had been fed with dried thyroid for 14 days or more invariably died within 60 seconds of an intravenous injection of pituitary extract which they had been able to withstand without obvious symptoms prior to thyroid feeding.

In order to study this apparent sensitizing effect of thyroid more closely 10 male rabbits of weights varying from 1·93 to 1·25 kg. were fed with 15 grains (0·972 g.) of a dried thyroid preparation daily for periods varying from 7 to 20 days. All of them had previously been shown to have no symptoms following intravenous injection of 0·3 c.c. of "Vasopressin" or "Pitressin"[1] beyond a slight degree of dyspnœa in some cases. At the end of about a week the rabbits were tested every 2 or 3 days with 0·2 c.c. "Vasopressin" or "Pitressin" intravenously and this dose was found to be fatal after a variable period of thyroid feeding.

It will be seen from Table I that there was considerable difference in

Table I.

Rabbit	Original weight (kg.)	Duration of feeding (days)	Weight at death	p.c. of weight lost
1	1·82	19	[1·35]	25·2
2	1·80	14	1·43	22
3	1·60	13	1·36	15
4	1·65	20	1·38	16·3
5	1·93	18	[1·36]	29·3
6	1·62	9	1·30	20
7	1·42	8	1·30	18·2
8	1·45	8	1·19	17·9
9	1·36	7	1·13	16·8
10	1·25	9	1·02	18·4

[1] "Vasopressin" and "Pitressin" are the trade names adopted by Messrs Parke, Davis and Co. for the pure pressor extract of the posterior lobe of the pituitary. I am greatly indebted to this firm for an ample supply of this extract.

the duration of thyroid feeding necessary to produce hypersusceptibility to pituitary extract; moreover, the susceptibility does not seem to depend on percentage loss of weight or on the relation between the lethal dose of extract and the final weight, for all animals had initially been tested with 0·3 c.c. of pressor substance while after thyroid feeding they succumbed to 0·2 c.c. Indeed, a great part of the weight lost is presumably relatively avascular fatty tissue, and therefore the area on which the pituitary extract acts (for the toxic action is on the vascular system) would probably be less diminished than the actual loss of weight indicates. Two rabbits (1 and 5) differed from the others in that after 19 and 16 days of thyroid feeding respectively, they showed no obvious increased susceptibility to pituitary extract; No. 5 after 16 days had lost 25 p.c. of its original weight and 2 days later was found dead; no explanation for this resistance has so far been discovered.

In all cases where death resulted the ears and the mucous membrane of the mouth were intensely blanched immediately after the injection and the animal had severe convulsions beginning within 20 seconds and lasting 20–30 seconds, followed by complete collapse; respirations, which finally were of a deep gasping nature, continued till death 60–90 seconds after the injection of pituitary extract, but at no time during the whole sequence of events was it possible to detect the heart beat by palpation. These points are of interest because Sharpey-Schafer(1) quotes several workers who state that the toxic action of pituitary extract is due to paralysis of the respiratory centre, while the experiments described here suggest that, after thyroid feeding at least, death is due to cardiac depression; this was subsequently confirmed by observations to be described below. In the normal anæsthetized rabbit, too, it can be shown that a dangerous degree of cardiac depression may be produced by intravenous injections of pituitary extract while respiration is little affected. That death after thyroid feeding was due to the pressor fraction of the pituitary extract was shown by the fact that no symptoms were produced in rabbits 7, 8, 9 and 10 by intravenous injections of 0·2 c.c. of the oxytocic fraction ("Oxytocin") even within 2 hours of the fatal dose of "Pitressin."

The action of pituitary extract on anæsthetized animals.

The blood-pressure changes in rabbits following an intravenous injection of histamine-free pressor extract of pituitary differ from those usually seen in cats in that the first injection produces a preliminary rise always followed by a definite fall which is succeeded by a rise, usually

of greater height and duration than the initial rise. Subsequent injections at intervals of 10–30 minutes give an increase in blood-pressure with little or no fall. It has been stated(3) that rabbits do not show tachyphylaxis to pituitary extract, but many experiments on rabbits anæsthetized with chloralose or amytal clearly show that this phenomenon is present (Fig. 1).

Fig. 1. Normal rabbit anæsthetized with amytal.

It is not so complete as in cats and is only seen in the course of the first three or four injections; thereafter with the same dose of pressor substance there is no further diminution of the rise in blood-pressure; but if an hour or more elapses between two small doses of pituitary extract the second response is more nearly equal to the first than if a shorter interval intervenes and the dip in the pressure curve is more evident. In some cases towards the end of a long experiment it may be found that repeated injections of pituitary extract produce responses in which a dip in the blood-pressure curve between two rises is a constant feature; in these cases however the dip is never so abrupt as that described above, and the explanation may be that the cardiac muscle has become impaired in the course of the experiment.

Records of ventricular contractions, taken by means of a thread attached to the apex through a window in the chest and to a heart lever, clearly show that the fall in blood-pressure referred to above occurs simultaneously with a slowing of the heart and a diminution in size of the individual contractions; during the first rise in blood-pressure there may be a transient increase in the amplitude of the heart beat, especially if the pressure is initially low, but at a point corresponding to the beginning of the fall in the carotid pressure there is often some cardiac irregularity, followed by rapid decrease in the size of the beat; slowing of the heart may persist till the highest point of the second rise in blood-pressure, although considerable recovery in the size of contraction has

taken place. Intestine oncometer records give no indication of a vaso-dilatation corresponding to the fall in blood-pressure (Fig. 2).

Fig. 2. Normal rabbit anæsthetized with chloralose. Both vagi cut. Artificial respiration. Upper record, ventricular contractions. Middle record, carotid pressure. Lower record, intestinal volume; fall of lever shows decrease in volume. At † 0·05 c.c. "Pitressin" intravenously.

In rabbits that have been fed on thyroid, the response to pressor extract is altered in that the fall in blood-pressure is greatly exaggerated

Fig. 3. Rabbit fed thyroid for 10 days; amytal anæsthesia.

(Fig. 3) and in fact may be so great that the animal does not recover, despite vagus section and cardiac massage (Fig. 4). It is also evident from Fig. 3 that repeated injections all produce a definite fall in blood-pressure as part of the response, which thus differs from that seen in normal animals, while the tachyphylactic phenomenon is less in evidence except between the first and second injections.

The cause of the cardiac depression is not clear; from previous work on different species of animals (3) it might be supposed to be due in some way to vagal inhibition, and one experiment in the present series seemed

to support this view. In this single instance the blood-pressure fell suddenly to 18 mm. Hg, and after 2 minutes, when the pressure was only

Fig. 4. Amytal anæsthesia. *L*, left vagus cut. *R*, right vagus cut. *AAAA*, cardiac massage.
Previous to thyroid feeding this rabbit, unanæsthetized, had shown no special reaction to six times this dose of "Pitressin."

2–3 mm. higher, the right vagus was cut without any change in pressure; but when one minute later the left vagus was cut the pressure immediately rose to 124 mm. Hg, to fall after 1½ minutes to a level a little above the initial level before the injection (Fig. 5).

A similar recovery has been observed in one experiment after the intravenous injection of 0·2 c.c. of 0·5 p.c. atropine sulphate followed by cardiac massage. Fig. 4, however, indicates an experiment in which section of both vagi and cardiac massage failed to cause recovery. Moreover, rabbits 7 and 8 in Table I both had the left vagus cut prior to beginning thyroid feeding without any apparent increase in resistance to pituitary extract. In normal rabbits under amytal or chloralose anæsthesia neither previous injection of 0·4 c.c. of 0·5 p.c. atropine sulphate nor section of both vagi (Fig. 2) eliminated the dip in the blood-pressure following pressor extract. It appears therefore that the vagus plays little if any part in this type of cardiac inhibition and that the examples given above, which seem to indicate the contrary, were acci-

dental; indeed, in the first case (Fig. 5) the blood-pressure showed a slight but gradual improvement before vagus section.

Fig. 5. Rabbit fed thyroid for six days.
0·55 c.c. "Pitressin" at †. *R*, right vagus cut. *L*, left vagus cut.

From what is already known of the action of pituitary extract, it is much more reasonable to suppose that the cardiac inhibition is due to vaso-constriction in the coronary circulation. I am indebted to Dr Anrep for the information that the pure pressor extract reduces the diastolic coronary inflow and that the rabbit's heart appears to be affected to a greater extent than that of the cat. This observation is in line with the fact that cardiac depression is very evident in the rabbit and slight or absent in the cat. If this explanation of the phenomenon is correct, the partial disappearance of cardiac depression after consecutive doses of pituitary extract at short intervals (Fig. 1) is explicable as part of the process of tachyphylaxis and indicates the necessity, in any investigation of the factors involved, of allowing a sufficient time for recovery from one injection to elapse before concluding that any drug or operative procedure prevents or reduces cardiac depression due to a subsequent injection of pituitary extract.

"Pitressin" in doses of 0·05 or 0·1 c.c., when given subcutaneously, does not produce the sudden cardiac depression characteristic of intravenous injection, nor does it cause a rise in blood-pressure comparable with that seen in the latter case, but four or five subcutaneous doses at intervals of ten minutes produce a degree of tachyphylaxis such that if

0·05 c.c. be given intravenously after a similar interval, cardiac depression is slight or absent. It is conceivable that the comparatively slow absorption of pituitary extract from the subcutaneous tissues reproduces in a crude way the effects of the normal secretion of the pituitary body; if this is the case then it is to be expected that if this rate of secretion is increased, the animal will be less susceptible to an injection of pressor extract because of the tachyphylaxis evoked by its own hormone. Conversely a greater degree of susceptibility might be expected where the secretion is diminished. It was thought that the explanation of the enhanced response of thyroid-fed rabbits to pituitary extract might be related to variation in secretion of the pituitary body, but published evidence of the effects of thyroid feeding on posterior lobe secretion is too indefinite to lend support to the hypothesis. It is known that thyroid feeding reduces the size of the pituitary gland, but the anterior lobe appears to be the part chiefly affected(4) and Herring states that the secretion load of the posterior lobe is not altered(5). There is, however, no evidence for or against an altered rate of secretion. It is hoped to throw more light on the problem in a future paper, in which the interference with carbohydrate storage will also be dealt with.

Summary and conclusions.

1. Feeding rabbits on dried thyroid gland increases their susceptibility to the toxic effects of an intravenous injection of pure pressor pituitary extract.

2. Normal anæsthetized rabbits show tachyphylaxis to the first three or four of a series of injections of pressor pituitary extract.

3. In thyroid-fed rabbits, and probably in the normal animal, death from pituitary pressor extract is due to cardiac depression and not to respiratory failure. This cardiac depression does not seem to be dependent on the activity of the vagi, and it is suggested that it is due to an action of the pressor extract on the coronary vessels in which the usual phenomenon of tachyphylaxis is shown.

I wish to express my thanks to Prof. Leathes for his helpful criticisms, and to the Medical Research Council for defraying expenses in this work.

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THE VASCULAR REACTIONS OF THE COLONIC MUCOSA OF THE DOG TO FRIGHT.

By A. N. DRURY, H. FLOREY and M. E. FLOREY.

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The experiments to be recorded originated in an accidental observation on a dog with a double Thiery fistula of the colon. One of the fistulæ presented an everted portion of mucous membrane which became very pale when the dog was at all alarmed, as for instance, by the sudden opening of a door.

The Thiery fistula type of preparation was modified as follows, in order better to investigate the phenomenon. Through a longitudinal midline abdominal incision in a dog previously purged with castor oil, the colon was withdrawn. Between clamps a portion of it, about 2 cm. long, was cut so as to preserve the mesenteric blood supply intact. Through a stab wound, conveniently placed, this portion was withdrawn completely from the abdomen, its clamps, which were of the crushing variety, removed and any fæces present wiped away. A crushing clamp was inserted longitudinally into the resected piece of gut so that the wall was crushed opposite the mesenteric attachment. This clamp was left *in situ*. It was then covered with a moist swab till later. An end-to-end anastomosis was then performed to restore continuity to the colon, which was replaced in the abdomen and the longitudinal wound sewn up. The portion of the colon withdrawn through the stab wound was then split longitudinally along the line crushed by the clamp left on during the performance of the anastomosis, and opened out to be sutured into a rectangular area from which the skin had been removed. A wet saline dressing was applied to the patch immediately after operation; subsequently euosol dressings were used daily and after three to four days vaseline was applied liberally to the patch which was then covered with a dry dressing. Such a preparation usually healed perfectly in ten days, though infection of the longitudinal wound occasionally gave trouble. The mucosa of such a healed patch was usually redder than that of an animal whose colon was opened during anæsthesia, a

result explicable by the continued exposure to air and friction of the bandages. Nevertheless the vessels remained quite reactive in spite of this dilatation. There is no tendency for the skin to replace the colonic mucosa, as a dog has been kept 11 months without diminution in size of the patch.

Reactions exhibited by the colonic patch.

In successful preparations it was found that any cause of alarm, such as a sudden rattling of a tray of instruments, banging on the table, or

Fig. 1. Section to show the junction of the skin and colonic mucosa at the edge of a patch.

sometimes the approach of a strange person resulted in a very rapid and extreme pallor extending all over the patch. The first unmistakable paling occurred 4 to 5 seconds after the stimulation and the full pallor lasted a variable time, some 5 to 10 seconds elapsing before the intensity of the reaction began to diminish, a return to normal following in about 20 seconds. A severe fright such as produced by the inhalation of a small quantity of ether for about 5 seconds initiated a most intense blanching which lasted 10 to 15 seconds. Under these conditions the patch would change in colour from a bright red to a dirty yellowish white when exhibiting a maximal reaction.

Further to analyse the mechanism underlying this reaction the following preparation was made. After the patch had completely healed (actually 43 days after the first operation), the abdomen was again opened and the pedicle of the patch tied off and cut, a procedure similar to that used by Ivy(1) for the production of an isolated stomach pouch. Immediately after the operation the patch was bloodless and cold, the extreme edges only appearing to have a blood supply. However, in the course of a day a collateral circulation from the skin and muscle opened up and the patch remained intact except for a slight superficial slough. Five days after operation it was observed to pale very slightly after a latent period of 15 seconds following the inhalation of a few drops of ether. It was thought that no vaso-motor nerves could have grown into the patch from the skin and muscle during the time which had elapsed since the cutting of the pedicle. The first conclusion was that the slight pallor observed was due to a humoral mechanism—in the light of Cannon's experiments to the liberation of adrenaline from the adrenals. It was however possible that the arteries of the skin and muscle, which were undoubtedly innervated, contracted and caused the slight pallor in the mucosa supplied by them. This suggestion was put to the test as follows. The abdomen of a cat, anæsthetized with chloralose, was opened and the colon located. The anastomoses headwards and tailwards of the inferior mesenteric artery were tied and the gut between opened longitudinally. The mucosa was of a bright pink colour. On obliterating the inferior mesenteric artery between the finger and thumb this pink colour became lighter—a pallor due simply to cutting down the arterial supply. The original colour returned immediately on release of the artery. It was clear then that the pallor of the isolated patch might be due to a constriction of the arteries of the skin and muscle supplying it.

Another point emerged from this experiment. A certain amount of pallor was produced by completely obliterating the inferior mesenteric artery. If, while still holding the artery, a drop of 1/1000 adrenaline chloride was applied, intense pallor resulted, although there was no arterial flow at the time. That is to say that in the very intense paling reaction to fear exhibited in the dog the smallest vessels which give colour to the mucosa are actively contracted. To determine where the vessels giving colour to the mucosa were situated, an injection of graphite ink was made into the inferior mesenteric artery of a cat. A portion, with the vessels ligated, was excised and fixed in formalin. After fixation the tissue was cut into strips about 1 mm. thick, dehydrated in alcohol

and cleared in clove oil. Before clearing, in a cross-section of the gut, the mucosa proper was of a dark greyish colour, while the submucosa presented a black line. After clearing it could be seen that the mucosa had a very rich supply of capillaries, while in the submucosa there was a rich plexus of larger vessels. A portion of mucosa viewed from the gut lumen surface presented a polygonal plexus of capillaries, having very much the appearance of endothelial cells, the borders of which were impregnated with silver. It is considered therefore that a considerable part of the colour of a colonic patch is due to the mucosa itself, while a certain proportion of colour is transmitted through the cells of the mucosa from the rich submucous plexuses.

Fig. 2. *Dog.* Showing colon patch and exteriorized spleen.

A further attempt was made to secure denervation of a colonic patch by a method other than cutting the pedicle. A dog was prepared in which the patch was taken from the part of the colon which was supplied by the inferior mesenteric artery and therefore presumably took its main sympathetic supply from the inferior mesenteric ganglion, the nerves from which accompany the artery. At a second operation all the nerves accompanying the inferior mesenteric artery were cut as they emerged from the inferior mesenteric ganglion, and the artery was stripped as thoroughly as possible. Though the time elapsing between stimulation and the appearance of pallor was lengthened from 5 seconds before to 10 seconds after the operation, it was found on a post-mortem histological examination that there were nerves still coursing with the vessels of the pedicle. In consequence the observations on this animal

after double adrenalectomy were of little value in determining to what extent the reaction was due to the liberation of adrenaline. However, another phenomenon, possibly of some significance in connection with the long-continued spasms frequently seen in the human colon, appeared. The day after the operation and subsequently for several days, the dog suffered from fits of the most intense tenesmus, some of which lasted hours, and succeeded in passing only a drop of liquid fæces after the most considerable efforts. In the course of about eight days, however, the dog apparently compensated in some fashion and began to pass normal-looking motions. The same phenomenon was found in another dog, though there was less evidence of tenesmus.

In two dogs an attempt was made to excise the inferior mesenteric ganglion and effect a thorough denervation and also insert the colonic patches at the same operation. Both animals, however, rapidly died of general peritonitis, and post-mortem examination revealed extremely contracted colons, the contraction apparently having been so vigorous that the end-to-end anastomoses had been torn apart, as the stitches in both the submucosa and muscle had been torn out in places. These observations point to the conclusion that the sympathetic innervation of the colon is of a tonic inhibitory nature. It has been customary to talk of the colonic spasms occurring in man as due to "over-action" of the nervi erigentes, but the "under-action" of the sympathetic supply would, as the above experiments show, offer as reasonable an explanation of the occurrence.

Efforts to produce a patch of colon which could be considered to be entirely denervated have not been successful, but the reactions of a fully innervated patch after double adrenalectomy have been satisfactorily observed. In this dog the adrenals were removed at intervals by the lumbar route. Four hours after the second operation the patch of this animal reacted to ether by an intense pallor, quite as intense as before the adrenalectomy, the latent period being 5 seconds. Twenty hours later the experiment was repeated with the same result—intense pallor and short latent period. Clearly, then, at least the main reaction involved in the pallor is nervous in origin. Whether any part of the reaction is of humoral origin, our experiments furnish no unequivocal evidence.

Reaction to mechanical stimuli.

If the mucous surface of a patch was struck with a small instrument or the tip of the finger, an intensely pale area, circumscribed to the region actually struck, appeared after about 1 second. This persisted a

variable time, sometimes for several minutes, depending on the severity of the mechanical stimulus. Even with the most severe stimulus, sufficient to cause slight hæmorrhage from the mucous surface, pallor always resulted. It had been seen from the experiment with adrenaline that the vessels giving colour to the colon were capable of independent contractility and from the observations upon capillaries elsewhere in the mammal one would conclude that this pallor to mechanical stimulation was due to the closing down of the capillaries. But in the gut it is possible that the contractions of the main gut muscles or the muscularis mucosæ might, during their contractions, squeeze the vessels in response to the mechanical insult and thus produce paling. Exner(2) noted, while studying the reactions of the alimentary canal against ingested sharp foreign bodies, that a prick with the needle was followed by a small circle of pallor, the centre of which was slightly indented. He attributed the pallor to contraction of the muscularis mucosæ forcing the blood out of the capillaries. But the capillaries may also react by contraction, as they do elsewhere in the body, to a light mechanical stimulus. However, the fact that very vigorous mechanical stimulation which elsewhere produces dilatation of vessels and reddening still brings about pallor in the gut suggests that the response to mechanical stimulation in the latter situation is due to the contraction set up in the smooth muscle fibres (the muscularis mucosæ) forcing the blood out of the vessels rather than to an intrinsic action of the small vessel walls.

That a reflex contraction occurring through the vaso-motor nerves in response to a tap is of importance in the pallor is ruled out by the following experiment. Cotton wool soaked in ½ p.c. ergotamine was applied to the patch until no pallor occurred on applying a drop of 1/1000 adrenaline. A tap, however, still readily produced a pale area[1].

Spontaneous changes in colour observed in dogs at rest.

These spontaneous changes in colour were best observed in a dog whose patch had been partly denervated, but they were observed in other dogs, though to a much less extent.

Careful observation of the patch showed that there were areas of pallor which were constantly changing their position, now several small

[1] Some experiments on the behaviour of lacteals also favour the view of the passive nature of the emptying of the vessels when mechanically stimulated. Microscopical observation has shown that if the gut wall be stimulated mechanically the lacteals visible are completely emptied of chyle during the contraction of the muscle, but that they fill *pari passu* with the relaxation of the muscle—clearly a case of passive emptying of vessels by contraction of smooth muscle in the vicinity(3).

discrete areas would be pale, now a larger area in some other part of the patch. In some cases the colour was seen to return to these areas suddenly from the edges in much the same way as the colour returns to the skin after expressing the blood with the finger. Mass movements of the patch due to contractions of the circular and longitudinal gut muscles were not necessarily associated with pallor, quite considerable puckerings of the patch occurring without alteration of colour. From the appearance of blood returning from the edges of the pale areas, it was thought that a large factor in the production of these spots was the passive expulsion of blood caused by contraction of the muscularis mucosæ. The fact also that the spots were best seen in a patch deprived of a considerable amount of its sympathetic nerve supply also favoured this view.

The pallor associated with fright invariably involves the whole of the patch uniformly, provided no areas of inflammation from irritation are present as these react much less or not at all.

Apparently there are two mechanisms present to deprive the mucosa of blood—one due to the active closure of the vessels, a reaction which is seen in response to fear, characterized by a uniform pallor and gradual return of colour, the other due to passive squeezing of the blood from the vessels by the contraction of the muscularis mucosæ of the intestine, which follows mechanical stimulation, characterized by the patchy nature of the pallor and return of colour from the edges.

These observations have a certain bearing on the James-Lange theory of the emotions. This theory supposes that a visceral reaction occurs in response to a stimulus and that this reacts on the higher centres producing the quality of the emotion. However, in these experiments the dog always clearly manifested the emotion of fear before the onset of any recognizable vaso-motor symptoms. Though the physiological evidence against the James-Lange theory is very considerable, the psychologists have been slow to recognize this, and these observations may be of service in providing one more piece of evidence against it.

A suggestion is also made that persons suffering from "nervous dyspepsia" may be victims of excessive visceral vaso-motor reactions, whereby their digestive glands are deprived of a considerable part of their blood supply as a result of the reactions to slight external stimuli.

SUMMARY.

1. A method is described for preparing a piece of colonic mucosa for observations on its vaso-motor and other reactions over extended periods of time.

2. These colonic patches in dogs react to various stimuli by a very pronounced pallor.

3. The pallor can be fully developed in the absence of the adrenal glands.

4. Localized pallor is produced by mechanical stimuli, a result attributed to the contraction of the muscularis mucosæ.

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THE EFFECTS OF EXERCISE ON THE VASCULAR CONDITIONS IN THE SPLEEN AND THE COLON.

By J. BARCROFT and H. FLOREY.

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Drury, Florey and Florey(1) described a preparation in which a patch of colon was exteriorized, the mucous surface replacing a portion of skin in the side of a dog. The patch is about 2½ cm. in diameter. It appears to maintain its normal character for a long time; one such dog we have kept in good condition for 11 months.

This preparation, as shown by Drury, Florey and Florey, readily shows any alteration in the vascular condition. Barcroft and Stevens (2) described a similar preparation of the spleen in which the more gross changes in volume in that organ may be observed.

There is no difficulty in making these two preparations on the same dog, and then comparing the effects of various procedures on the spleen and the mucous surface of the colon respectively. Our object in the present case was to compare the effects of exercise on the turgidity of the two organs.

The effect of the intestinal operation on the size of the spleen.

Two dogs were used during the present research. The first, "Tipsy," had its spleen exteriorized on June 4th and the colon operation performed on August 1st, 1928. In the case of the second dog, "Betsy," the procedure was reversed, the colon operation being performed on August 8th, 1928, and the spleen exteriorized on October 18th. The case of Tipsy yielded an opportunity of observing the effects on the size of the spleen of an operation on some other abdominal viscus.

Table I gives the area of the projection of the spleen on a surface of celluloid overlying the organ.

Table I.

Days *before* operation on colon	27		25		16		9		7		During operation				
Area in sq. cm.	36·5		39		43		37·5		40		22				
Days *after* operation on colon	5	6	7	8	16	24	33	38	44	52	58	66	67	88	
Area in sq. cm.	24	21·5	22	21	20	20	23	30	28	39	29	31	27	12 (dead)	

In Fig. 1 the approximate alterations in volume are shown. The volume of the spleen shortly before the operation is taken as 100. On

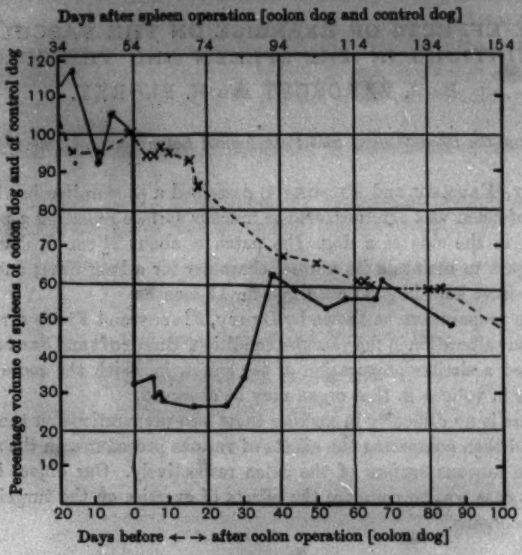

Fig. 1.

the figure are also shown by a broken line the corresponding alterations in volume for a control dog. The "100" volume for it is taken on the same number of days after the exteriorization of the spleen as that for the dog on which the colon experiment was performed. That the spleen should have shrunk temporarily during the operation is normal. The administration of chloroform or ether is known to produce that effect, and this has frequently been verified by one of us. The remarkable thing is that the spleen should have remained shrunken and practically bloodless for a month subsequently. It then distended and settled down to a volume about twice what it had been immediately after exteriorization of the colon patch and about 60 p.c. of what it was before the operation.

It seems premature to discuss the cause of these alterations until more is known about the mechanism. The first step, and one which is being already taken, is to repeat the experiment on denervated spleens,

a procedure which should go some way to show whether the contraction of the spleen is or is not caused by the absorption of some material from the seat of the operation.

Presumably, as the spleen has not been tampered with directly, the contraction must be due either to chemical or nervous impulses of an abnormal character reaching it from without as the result of the operation on the intestine.

It is not only this particular operation under discussion which produced semi-chronic contraction of the spleen. Figs. 2 and 3 show the

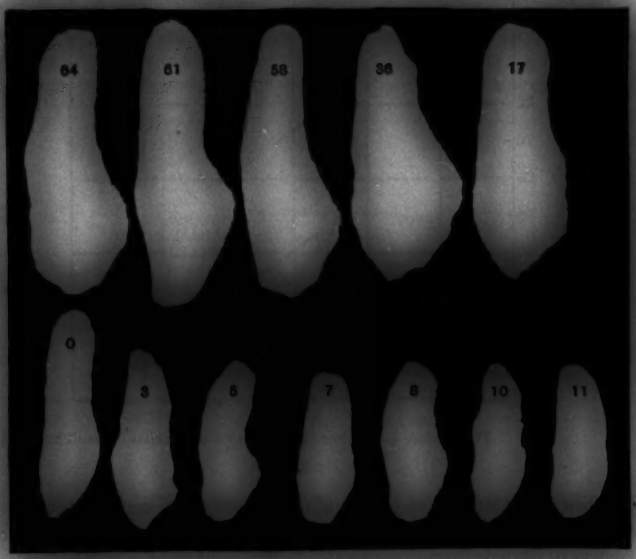

Fig. 2. Variations in the size of the exteriorized spleen due to exteriorization of the portion of jejunum. The figures indicate the number of days before or after the operation on the intestine.

same phenomenon in two animals which had portions of the jejunum exteriorized. Neither of these animals lived till the spleen returned to its relaxed condition. The first remained in excellent health until 25 days after the exteriorization of the portion of small intestine. It then lost

appetite and on the 27th day it developed an intestinal fistula and was killed. The fistula was due to the perforation by a long piece of bone which it had swallowed unknown to us.

Fig. 3 is the record of an animal (1) in which a portion of small

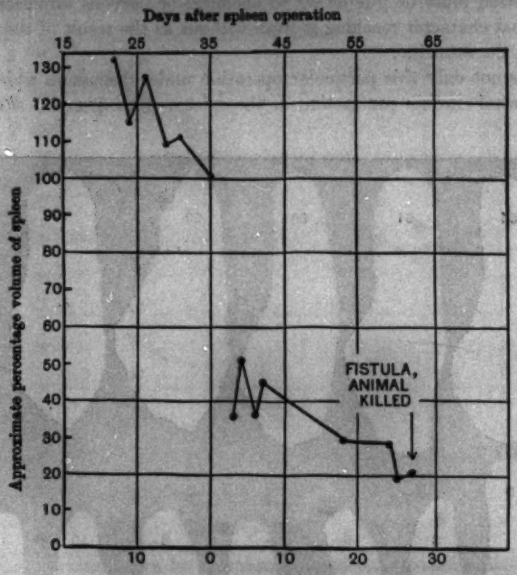

Fig. 3.

intestine was exteriorized on the 35th day after exteriorization of the spleen, and which (2) was killed 27 days subsequently and found *post-mortem* to have developed peritonitis as the result of an abscess.

To return, however, to the effects of exercise on the exteriorized spleen and colon patches of Tipsy and Betsy. The size of the spleen was observed by the method already described, the more difficult observation was that of the patch of colon. For this purpose an artist (Mr Gillings) made us a series of colour standards somewhat after the manner of the Talquist Blood Standards. Indeed the Talquist Standards might have sufficed had they been, apart from intensity, the same tint as the spleen.

The standards which we used ranged from just redder than the colon was observed to be, to a pallor little removed from cream colour. This range was divided into ten standards. These of course mean nothing in units, but they proved more satisfactory than hæmoglobin mixtures of various concentrations and other devices which we tried.

In the case of Tipsy, the spleen having been exteriorized on June 4th and the colon patch on August 1st, the first observations were made on October 6th and though correct they proved to be quite misleading—the exercise taken was once up and down the laboratory stairs. The spleen as usual shrank but no change could be seen with certainty in the exteriorized gut.

An experiment of much shorter duration however was more illuminating. In it the exercise consisted simply in running in the laboratory passage, which took about 17 seconds. Fig. 4 shows a typical record

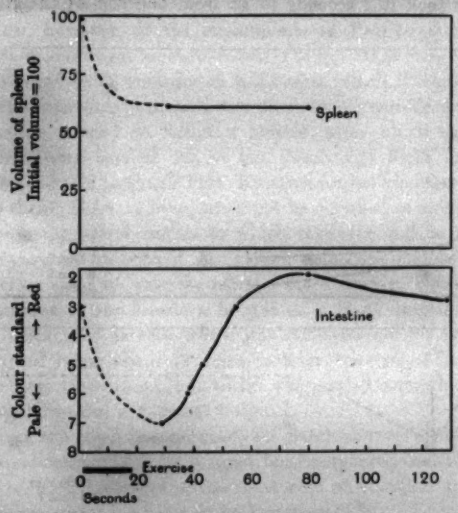

Fig. 4. Tipsy. Effect of short "sprint" on the volume of the spleen and the colour of the mucous membrane of the colon.

of such an experiment. The mucous membrane of the colon at first becomes intensely pale (7 on standard) and the spleen contracts, but 60 seconds after the onset of the exercise the intestine has become as

red as, if not more red than, it was when the animal was at rest. The spleen on the other hand remained shrunken. This experiment was repeated a number of times and always with the same result. Taking it together with the result obtained on October 6th and others of the same sort, it became necessary to study the effects of exercise extending over various lengths of time. For this purpose Betsy was used—her colon patch had been exteriorized on August 8th and her spleen on October 18th, 1928; the experiments detailed were made in the spring of 1929.

The first series of experiments to be cited were made on the roof of the Pathological Laboratory; here it was possible to get an open stretch about 33 yards in length. A person whose call the dog was trained to obey was posted at each end of the roof and the animal was made to run to and fro, as soon as it reached one end being called from the other. Roughly speaking it took it 4 seconds to go from one end of the roof to the

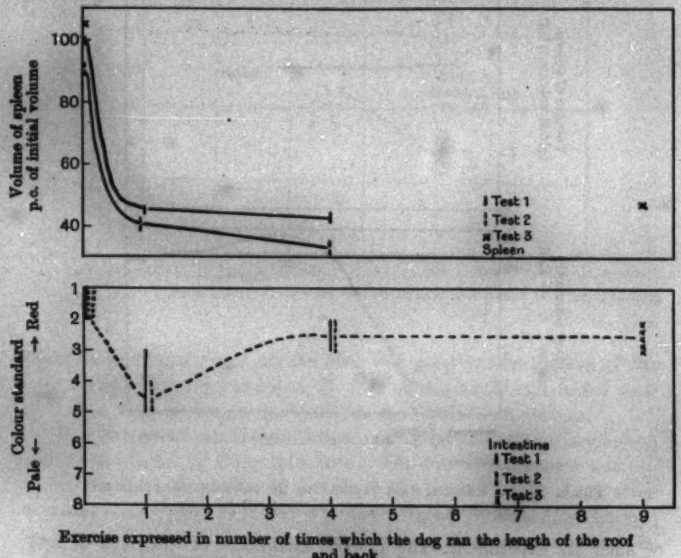

Fig. 5. Effect of more protracted exercise than shown in Fig. 5 on the volume of the spleen and the colour of the mucous membrane of the colon.

other, but the time was not recorded and the duration of the exercise is recorded in Fig. 5 in terms of the number of times which it went from end to end of the roof. This figure shows the result of tests on three separate occasions. On two, the dog was made to run (*a*) once and (*b*) four times the length of the roof, and on the third, nine times that distance. The result was a contraction of the spleen which persisted over the duration of the experiment, and a pallor of the intestine which was striking after the end of the first "lap" but was scarcely perceptible either at the end of the fourth or at the end of the ninth. These experiments bore out the earlier ones on Tipsy in showing that over a range of exercise covering not more than a few minutes the principal pallor of the patch of colon mucous membrane is only in the first stages and commences to wear off after the first 20 seconds or so and at the end of a minute is gone. The spleen however maintains its contracted condition as long as the exercise lasts.

In view, however, of the experiments of Pembrey and his co-workers (3), who brought forward evidence to show that on severe exercise there was considerable constriction in the renal vessels, it seemed desirable to carry out our own experiments over much longer periods of time. Betsy was therefore trained to follow a bicycle, after which the following experiments were carried out at the Cambridge Field Laboratories. Here we would like to thank Prof. Buxton for his kindness in allowing us to use them. These experiments must be regarded as preliminary, but they gave some useful results to which reference will be made later, therefore they are mentioned; their weak point was that as the animal became more tired its exercise became more uneven, therefore it was further trained to run beside a bicycle on a lead. In this way it was given an amount of exercise which was sufficient but not excessive, the average speed being about 12 miles an hour, covered in 20 minutes at a more or less even speed, but which was graded somewhat according as the dog did or did not appear fatigued.

So far as the colon is concerned, the patch showed a little, but only a little, vaso-constriction, for it must be remembered that although the colon on each of the above occasions started at standard 1 it was not at all uncommon for it at rest to be 2 or even 2–3.

As regards the spleen it contracted quite unequivocally and remained contracted not merely during the exercise but for at least as long (about 20 minutes) as the dog was subsequently under observation. The long duration of the contraction of the spleen after the exercise is complete, in cases of extended and strenuous exercise, is a new point.

The following are the results of two experiments:

TABLE II.

Period	Time	Colour of colon, standard	Remarks	Area of spleen sq. cm.	Approximate volume of spleen as a percentage of volume at rest
March 12					
1.	3.0 p.m. 11 min.	1 3–4	Exercise commences	13·5	100
2.	18 min.	2–3	Exercise less vigorous than in period 1	—	—
3.	20 min.	3	Exercise same as period 2	—	—
4.	30 min.	4	Exercise less vigorous than periods 2 and 3 but made a sudden spurt when let off lead	10·2	59
5.	50 min.	1	Period 5 resting in car	11·7	75
March 11					
1.	3.0 p.m. 10 min.	1 3–4	Exercise commences	—	—
2.	20 min.	3–4	Exercise ends	—	—
3.	37 min.	1	Period 3 resting in car	Obviously still much shrunk	

It has been noted elsewhere that the vessels of the colonic patch constrict when the dog is frightened or excited in any way. One dog, which was particularly attached to the laboratory attendant, exhibited the paling reaction when the latter left it to go out of the room.

To what extent then is the pallor associated with sudden exercise the result of "anxiety" or excitement?

That excitement plays at least a part is shown by the following experiment. The dog, held in such a position that the patch could be observed, was called from a distance. The patch became pale—about 4 on the standard. If now the dog were allowed to run the patch became still paler—7 on the standard. This however does not prove that part of the pallor was due to excitement and part to the actual exercise, as the dog when held did not display the very great degree of eagerness to run that it did when freed. During a demonstration to the Physiological Society the dog was made to run along a passage 25 yards long. After each of the first few runs the patch exhibited its usual reaction, but towards the end of the demonstration the patch reacted less and less until very little pallor was observed after the 25-yard run. The dog was then apathetic and took less interest in the proceedings than at the

beginning of the demonstration though it ran at much the same rate. It would be reasonable to attribute at least part of this lessening of reaction to loss of interest on the part of the dog. We feel justified in thinking, therefore, that the pallor exhibited after a short burst of exercise is in part the result of "excitement" of the dog.

It has also been found that the colon patches are very sensitive to mechanical stimuli which produce local areas of pallor. It was possible that during the running the patch was stretched or knocked by the hind leg. However, if the patch were stretched by pulling on the surrounding skin no pallor could be produced. To obviate the effect of accidental knocks a ring of cork, of sufficient depth to prevent contact of the patch with other parts of the body, was fixed round the mucosa by means of sticking-plaster. During running it was then impossible for mechanical stimulation to affect the colour of the mucosa but a paling reaction after a short run still developed in its full intensity.

SUMMARY.

1. The vascular reactions simultaneously exhibited by the spleen and the colon in dogs are described.

2. Short bursts of running result in pallor of the colon and contraction of the spleen.

3. During exercise of longer duration the spleen remains contracted while the colon exhibits only slight evidence of vaso-constriction.

4. The spleen remains contracted for a considerable length of time after moderately heavy exercise.

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AN IMPROVED TYPE OF PUMP FOR ARTIFICIAL RESPIRATION.

By A. R. SMELLIE.

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This pump differs from those described by Starling(1) and by Taylor and Olmsted(2) in that it possesses a mechanically operated quick-acting piston valve which may be attached to an older form of pump, such as Brodie's, after slight alteration.

In a single cylinder moves a trunk piston operated by a connecting rod and crank, the latter being slotted to allow rapid alteration of throw of the piston. The valve is operated by an eccentric on the crank shaft and is unaffected by alteration in the stroke of the piston.

The valve itself consists of an outer brass casing to which are fixed the inlet and outlet tubes and the connection to the cylinder. Inside the casing moves a piston valve in which the necessary ports are cut (Fig. 1).

The pump takes in air or a gaseous mixture by the air intake (Fig. 1 *A*). The valve remains in this position without movement until the piston is at the outer "dead centre," that is until the piston is on the point of changing from suction to compression. During this period too the animal expires, by the elasticity of its thorax and lungs, through a separate channel (Fig. 1 *A*).

Whenever the movement of the piston is reversed the valve changes suddenly to the position shown in Fig. 1 *B* and the cylinder contents are driven into the animal's lung. There is no connection between the pathway for expired and inspired air except by means of a Y-tube at the trachea. Thus the "dead space" of the respiratory tract is not increased.

The sudden change in valve position is brought about by a spring link motion. The lower half of this link motion is connected to the eccentric on the crank shaft, the upper half to the piston valve. The upper half of the link motion, together with the valve, remain stationary until the lower half of the motion has been carried by the eccentric rod beyond the same straight line as the upper half of the motion. The springs, which

have gradually been stretched during this period, now suddenly come into action and snap over the upper half of the link motion, carrying the

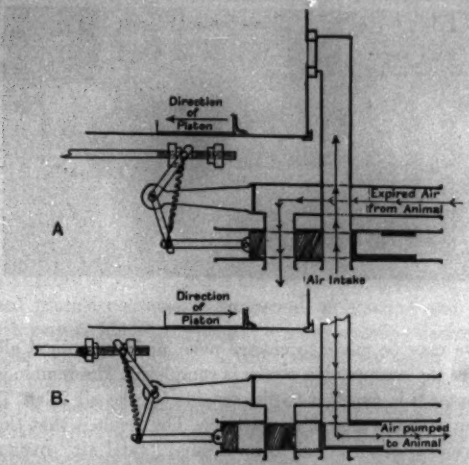

Fig. 1. *A*. Animal expiring, pump taking in air; *B*. pump delivering air to animal, intake and expiration ports closed.

piston valve, into the new position (Fig. 1 *A* and *B*). The valve returns to its previous position by a similar sudden action. By adjustment of the collars on the eccentric connecting rod the sudden flick over of the valve is timed to coincide with the inner and outer "dead centre." Thus the ports for exit or for entry of air remain fully open while the air is passing and there is no "strangling" of the air current by a gradual cut off.

Air of any desired composition may be delivered to the animal by attaching the air intake to a container with the necessary mixture of gases. The expired air may also be collected. The pump, motor, and reduction gear are mounted on a board and the entire mechanism may be carried with ease by one man (Fig. 2).

In the normal animal the inspiratory period is less than half of the total respiratory cycle. The usual type of mechanically operated pump cannot reproduce this cycle, the period of inspiration of necessity being equal to that of expiration.

This difficulty can be obviated however in the present form of pump. By adjustment of one of the collars on the eccentric connecting rod the

Fig. 2.

piston valve may be made to change over, and the animal allowed to expire before the compression stroke is completed. The remainder of the cylinder contents is expelled to the outside by the air inlet. This does not interfere with the animal's expiration. The result is that the animal obtains a volume of air equal to its normal "tidal air" over a period corresponding to its normal inspiration and expels that volume over a period corresponding to its normal expiration.

SUMMARY.

1. An existing pump of older form can be converted with ease to this type.
2. The stroke volume may be varied within all necessary limits.
3. The valve movement from position to position is sudden, is independent of the amplitude of movement of the trunk piston, and may be timed to fall at different periods of the compression stroke.
4. There is no increase in "dead space" for the animal.
5. The normal respiratory cycle may be mimicked perfectly.

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A METHOD OF ESTIMATING PHOSPHAGEN AND SOME OTHER PHOSPHORUS COMPOUNDS IN MUSCLE TISSUE.

By GRACE PALMER EGGLETON and PHILIP EGGLETON.

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The exact nature of phosphagen is not yet known. A protein-free extract of striated muscle contains a compound of creatine and phosphoric acid which may be identical with but is more probably derived from the phosphagen originally present in the muscle. It seems advisable to retain the term phosphagen for the substance existing in the muscle, reserving the chemical description creatinephosphoric acid for the substance isolated from acid extracts of muscle. Mr H. V. Horton, working in this laboratory, has shown that although phosphagen cannot diffuse from a living muscle, creatinephosphoric acid can diffuse readily through collodion membranes. This is true whether the creatinephosphoric acid has been extracted from the muscle by means of acid, dilute alkali, or alcohol. The inability of phosphagen to diffuse from living muscles may be due to the selective impermeability of the muscle cells to the creatinephosphoric anion, but it seems more justifiable to suppose that creatinephosphoric acid exists in the muscle in combination with some colloidal substance. Certainly the view that creatinephosphoric acid exists as such in the muscle, and that its disappearance in fatigue is a result of simple hydrolysis, raises serious difficulties. Meyerhof and Lohmann(1) have shown that creatinephosphoric acid has a large heat of hydrolysis, about 11,000 calories per gram molecule, but the total heat of contraction of a muscle has the same value relative to the lactic-acid production whether much or little phosphagen disappears simultaneously. Consideration of the myothermal data available leads us to the conclusion that the "breakdown" and "resynthesis" of phosphagen in a living muscle are reactions involving very little energy. (See A. V. Hill(2).)

The only available means of determining the amount of phosphagen in muscle is to estimate the creatinephosphoric acid in a protein-free

extract of the muscle. In the paper in which the presence of phosphagen in striated muscles was first reported (Eggleton and Eggleton(3)), we described a method for its estimation which was an elaboration of the colorimetric method of Briggs for estimating inorganic phosphate in tissues. This method has since been improved in certain respects, and as it is still the most rapid and convenient method for ordinary purposes, we take this opportunity of recording the improvements before describing the new method which is designed primarily for certain special cases.

Direct method.

Three stock reagent solutions are kept: (*a*) 5·5 *N* sulphuric acid (15 p.c. by volume), (*b*) 5 p.c. ammonium molybdate, and (*c*) 0·5 p.c. hydroquinone in 15 p.c. sodium sulphite ($Na_2SO_3 7H_2O$). The reagent used in an analysis is a mixture of two parts of (*a*), two of (*b*), and one of (*c*). This mixture is stable for 24 hours, but is in practice never kept more than 6 hours. 5 c.c. portions are added simultaneously to a standard phosphate solution and to the trichloroacetic acid extract of a muscle (both containing about 0·08 mg. of directly estimable phosphorus and having a volume slightly less than 10 c.c.), the volumes are rapidly adjusted to 15 c.c. and the solutions mixed. By reading the standard against the unknown in the colorimeter (the reverse of the usual procedure) the readings plotted against the time form a straight line for the first 8 minutes, and extrapolation backwards to zero time may be performed with a ruler. Theoretically two points only on this line are necessary to define its position: actually three sets of readings are made, at 2, 4, and 6 minutes, and if these do not fall on a straight line (as happens very rarely) the determination is repeated. A colorimeter one side of which is fitted with a rotating drum graduated in tenth-millimetres is more convenient than one fitted with the usual vernier scale.

The method as outlined above is accurate provided the ratio of orthophosphate to phosphagen phosphate is less than 6, and greater than 0·5, but cannot be used with confidence for well-oxygenated resting muscles in which the ratio may be 0·2 or for very fatigued muscles which may contain only a trace of phosphagen. The following device increases the range of applicability of the method to resting muscles. A quantity of the muscle filtrate expected to contain 0·1 mg. of directly estimable phosphorus is treated simultaneously with a standard containing 0·03 mg. of phosphorus (as KH_2PO_4) and the time comparison made between these two. A second standard containing 0·1 mg. of phosphorus is treated

in the course of the next few minutes, and is used for the final comparison (90 minutes).

In Table I are given the analyses (performed in collaboration with

TABLE I. Orthophosphate and phosphagen contents of excised muscles maintained in oxygen and killed in ice-cold trichloroacetic acid.

Exp.	Muscle	Duration of exposure in oxygen (hours)	Orthophosphate	Phosphagen	Ratio
1.	Sartorius	2	10	76·5	0·13
2.	Sartorius	2½	14	57	0·24
3.	Sartorius	1	16·8	58·7	0·29
4.	Sartorius	1	15·1	71·9	0·21
5.	Sartorius	2½	15·7	65·3	0·24
6.	Sartorius	2	20	61·5	0·32
7.	Gastrocnemius	20	19·5	74·5	0·26
8.	Gastrocnemius	?	16·7	52·5	0·32
9.	Gastrocnemius	?	14·1	53·3	0·26
10.	Gastrocnemius	12	18·5	74·5	0·25

In *Exps.* 1–7 the Hungarian giant frog was used, in the last three the Dutch frog (*R. esculenta*). In *Exp.* 5 the muscle was first fatigued. Results are given in mg. of phosphorus per 100 g. of muscle.

Mr H. V. Horton) of several muscles which had been kept in oxygen for periods ranging between 1 and 20 hours. Such muscles are rich in phosphagen and frequently contain as little as 15 mg. of orthophosphate phosphorus per 100 g. of muscle. It seems not unlikely that resting muscles in the intact animal may contain even less orthophosphate. It was in the course of these experiments that Mr Horton first observed the condition of reversible inexcitability in voluntary muscle (Dulière and Horton(5)). In view of these analyses and the experiments of Sacks and Davenport(6), in which similar low values were obtained for warm-blooded animals, it seems likely that many workers on this subject are using faulty technique; for the orthophosphate values quoted for "resting" muscles are frequently higher than the phosphagen values. It seems not unlikely that the true orthophosphate content of a resting muscle in any animal is little, if any, higher than that of the blood. Stella has shown, for example, that resting frog muscles are in diffusion equilibrium with a physiological saline solution containing 8 mg. p.c. of orthophosphate phosphorus, a value only a little higher than the orthophosphate content of the blood of most vertebrates (Stella(7)). For present purposes however it is sufficient to note that in the case of really resting muscles a modified technique such as has been suggested above is essential.

For most purposes the above method is both convenient and accurate, but in certain circumstances it is valueless. When it is necessary to decide whether a tissue contains a trace of phosphagen or none whatever (a matter which is sometimes of great theoretical interest) it is essential to use some other means of estimating the phosphagen, and the following technique was designed primarily to meet this case.

New method.

This method is based on our earlier observation (Eggleton and Eggleton(4)) that barium creatinephosphate is soluble in water: the extreme insolubility of barium phosphate should lead, and does lead, to a clean separation of the two salts. The following procedures are involved.

1. Extraction of the acid-soluble phosphorus from the muscle sample with $N/4$ trichloroacetic acid.

2. Neutralization of the extract with finely powdered solid baryta to a *p*H of approximately 9. This precipitates barium ortho- and pyrophosphates completely, but leaves the creatinephosphate in solution.

3. Separation of the precipitate from the soluble barium salts (fraction *A*) by centrifugalization.

4. Solution of the washed precipitate in HCl (fraction *B*).

5. Estimation in fraction *A* of

(*a*) directly estimable phosphorus (creatinephosphoric acid),

(*b*) total phosphorus.

Estimation in fraction *B* of

(*c*) directly estimable phosphorus (orthophosphate),

(*d*) phosphate hydrolysed to orthophosphate by seven minutes' boiling in $N/1$ HCl (pyrophosphate according to Lohmann),

(*e*) total phosphorus.

The successive stages of this technique are discussed in detail below.

Detailed discussion of new method.

Stage 1. The muscle, weighing 0·5 to 1·5 g., is ground thoroughly in 5 c.c. of $N/4$ trichloroacetic acid with acid-washed sand; muscle, acid, and mortar having been cooled to 0°. Further trichloroacetic acid is added from a burette to make a total of 10 c.c. per g. of muscle used. The mixture is centrifugalized and the extract poured through a filter paper (phosphate free) into a test-tube, and a suitable volume is pipetted into a dry centrifuge tube. The procedure of centrifugalization followed by filtration is more rapid and efficient than either alone. If the muscle

be assumed to contain 85 p.c. of water the proportion of acid-soluble phosphorus extracted is represented by the ratio of the volume of extract taken to the total volume of water in the system. Thus if the muscle weighs 1·15 g. and the volume of extract taken is 10 c.c. the proportion taken is 10/(11·5 + 85 p.c. of 1·15) or 80·5 p.c. of the acid-soluble phosphorus of the muscle. That this is true for the orthophosphate, labile phosphate and total phosphate is shown by the results in Table II.

TABLE II.

Exp. 1. 2·7 g. of muscle were ground with 13 c.c. of 4 p.c. trichloroacetic acid and centrifuged. The extract was poured off and measured. The residue was extracted four times in succession with 8 c.c. of acid, each extract being measured. The five extractions took 25 minutes. The figures quoted for inorganic and labile P have been corrected for the known rate of hydrolysis of creatine-phosphoric acid in 4 p.c. trichloroacetic acid.

Volume of acid used to extract	13·0	8·0	8·0	8·0	8·05 c.c.
Volume of extract	12·6	7·65	8·0	7·9	7·9 c.c.
Proportion of acid-soluble phosphate in each extract. Theoretical*	82·4	12·6	3·6	1·0	0·3 p.c.
Do. Actual	82·3	12·5	3·4	0·7	0·2 p.c.
Proportion of inorganic phosphate	27	25	—	—	—
Proportion of labile phosphorus	36	36	—	—	—
Proportion of remainder	37	39	36	—	—

Exp. 2. As above: weight of muscle 3·78 g.

Volume of acid used to extract	15·0	10·0	10·0	10·0 c.c.
Volume of extract	12·4	12·15	10·15	10·15 c.c.
Proportion of acid-soluble phosphate in each extract. Theoretical*	67·0	24·0	5·6	1·55 p.c.
Do. Actual	67·1	24·0	5·6	1·6 p.c.
Proportion of inorganic phosphate	31	30	30	—
Proportion of labile phosphorus	26	26	25	—
Proportion of remainder	43	44	45	—

* Assuming 85 p.c. water in muscle, and instantaneous extraction. Acid-soluble phosphorus remaining in the protein precipitate at the end was extracted overnight with 10 c.c. of trichloroacetic acid.

The agreement between theoretical and the observed extraction in each successive extract is good. Moreover, the extraction must be complete in a few minutes[1], for the theoretical values quoted in the table assume instantaneous extraction.

Stage 2. The trichloroacetic acid extract in the centrifuge tube is neutralized by the addition of finely ground baryta (best sifted through a 90-mesh sieve) until neutral to phenolphthalein. A small final adjustment is occasionally necessary with *N*/4 trichloroacetic acid or saturated baryta solution. The presence of some carbonate in the powdered baryta is of no consequence. As far as this stage the operations are carried out

[1] The contrary experience of some other workers (*e.g.* Irving and Wells(11)) seems to be due to less thorough grinding of the tissue.

as rapidly as possible to minimize hydrolysis of creatinephosphoric acid, but when neutralized the solution may be left, and is indeed perhaps better left, for a short while before the next operation.

Stage 3. The mixture is centrifugalized to remove the precipitate of insoluble barium phosphates, the clear fluid being decanted into a measuring cylinder. The precipitate is washed with 2 c.c. of $N/4$ barium trichloroacetate neutral to phenolphthalein, centrifugalized, and the washing added to the soluble fraction. No further washing is usually made, for such washings merely extract minute quantities of some phosphoric ester which is almost but not quite insoluble in the reagent used. Water may not be used for this washing, since it dissolves appreciable quantities of orthophosphate.

Stage 4. The precipitate is dissolved in a drop of $8N$ HCl and washed into a second calibrated cylinder, the successive washings totalling about 10 c.c. of water. The volumes of both fractions are measured.

The use of measuring cylinders at this stage is very convenient and involves no loss of accuracy if the cylinders have been calibrated against a standard burette. We use 25 c.c. cylinders graduated in one-fifth c.c., calibrated at each 5 c.c. mark. Only cylinders with constant and small errors are retained and are used in conjunction with a calibration chart. We have satisfied ourselves that the use of these cylinders, although unorthodox, is not a source of error.

Stage 5. The acid-soluble phosphorus of the muscle is estimated in five fractions, of which two have soluble barium salts. The soluble fraction contains all the creatinephosphoric acid but no orthophosphate. This is shown by the following facts:

(i) Barium orthophosphate is found to be completely insoluble under the prescribed conditions: 10 c.c. of fluid in equilibrium with a barium phosphate precipitate contains no phosphorus that can be detected by the Briggs method.

(ii) The colour production curve of the soluble fraction, followed under the conditions described earlier in this paper for use with resting muscles, starts from the origin (Fig. 1).

(iii) A muscle in an advanced state of rigor which contains much orthophosphate gives no directly estimable phosphorus in the soluble fraction (Table XI). An acid extract, made from any muscle and allowed to stand until all the creatinephosphoric acid is hydrolysed, behaves similarly.

(iv) Orthophosphate added to a muscle extract is recovered quantitatively in the insoluble fraction (Table IV).

(v) Further extraction of the insoluble barium salts with the prescribed extracting fluid extracts no directly estimable phosphorus.

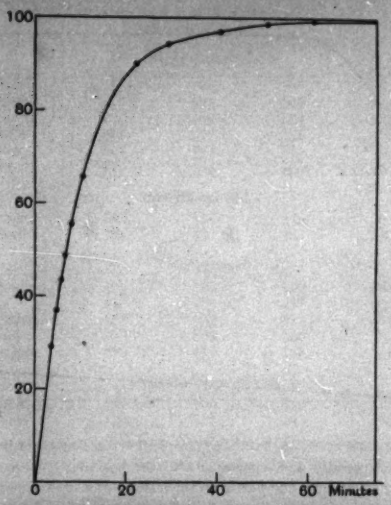

Fig. 1. The phosphagen fraction of a muscle extract prepared according to the text was compared with pure orthophosphate solutions in respect of the rate of blue colour production after the addition of Briggs reagents. The curve gives the change of colour intensity of the phosphagen solution relative to the inorganic phosphate standard as time progressed. The colour values are expressed as a percentage of the value finally attained (100 minutes). The first six points were obtained by a comparison with a phosphate standard having a value 37 p.c. on the above scale, the last five points with a standard of value 88 p.c. Note that the curve starts from the origin and is practically a straight line for the first 8 minutes.

The organic phosphorus in this fraction includes any barium hexosemonophosphate present in the muscle, together with any other phosphate esters with soluble barium salts. Whatever be the compounds included here, it seems justifiable to class them as a distinct fraction since the separation from the insoluble salts is so nearly complete.

With regard to the "pyrophosphate" fraction we have not ourselves attempted to isolate this compound, but we have found that:

(i) Pyrophosphate is completely hydrolysed by the treatment given.

(ii) A similar hydrolysis curve is given by the solution obtained

from muscle, when allowance is made for a very gradual hydrolysis of some other, relatively resistant, ester (Fig. 2 and Table III).

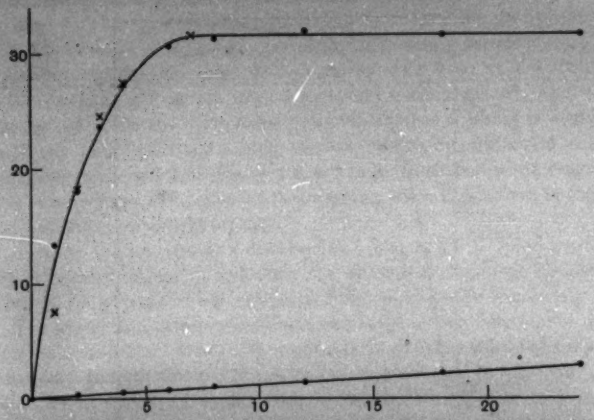

Fig. 2. The hydrolysis curve of the "pyrophosphate" fraction given in Table III is here reproduced graphically and compared with the hydrolysis curve of pyrophosphate under similar conditions. The points on the latter curve are marked with crosses. The lower almost horizontal line represents the slow hydrolysis of some phosphoric ester. The ordinates are mg. of phosphorus per 100 mg. of muscle, the abscissæ time in minutes.

TABLE III. Hydrolysis of fraction with insoluble barium salts in $N/1$ HCl at 100° C.

Time in minutes	Orthophosphate present	Orthophosphate produced	Analysis of compound hydrolysis curve	
			"Pyro-phosphate"	Other phosphorus
0	32·5	—	—	—
1	45·9	13·4	13·3	0·1
2	50·5	18·0	17·7	0·3
3	56·2	23·7	23·3	0·4
4	59·9	27·4	26·9	0·5
6	63·2	30·7	30·0	0·7
8	64·8	32·3	31·3	1·0
12	65·8	33·3	31·9	1·4
18	66·3	33·8	31·6	2·2
24	67·1	34·6	31·7	2·9

The experimental hydrolysis curve analyses directly into two curves, one of which is identical with that of pyrophosphate (Fig. 2). The other is practically a straight line, and registers the very slow hydrolysis of some phosphoric ester. The results are expressed in mg. of P per 100 g. of muscle. The total phosphorus in this fraction was 81·6 mg. per 100 g. of muscle.

(iii) Pyrophosphate added to the original muscle extract appears quantitatively in this fraction (Table IV).

TABLE IV. Recovery of ortho- and pyrophosphate added to trichloroacetic acid extracts of muscle.

Experiment	1	2	3	4	5
Original analysis.					
Orthophosphate	515	595	406	475	491
Phosphagen	231	142	254	313	384
Pyrophosphate	313	292	236	268	317
Soluble esters	144	109	141	95	95
Insoluble esters	180	143	145	142	172
Phosphorus added.					
Orthophosphate	188	188	218	220	227
Pyrophosphate	134	137	137	130	129
Final analysis.					
Orthophosphate	701	795	620	696	720
Phosphagen	240	139	257	306	373
Pyrophosphate	452	431	378	401	450
Soluble esters	135	106	132	85	91
Insoluble esters	180	142	134	151	180
Phosphorus recovered.					
Orthophosphate	186	200	214	221	229
Pyrophosphate	139	139	142	133	133

The extract from a muscle sample was divided into two parts, to one of which was added a known quantity of pyrophosphate and orthophosphate. The effect of this alteration in the distribution of the different phosphorus fractions is shown in the table. Results are given in micrograms of P per g. of muscle.

The five phosphorus estimations involved in a full muscle analysis are carried out as described below.

Fraction A.

(*a*) Directly estimable phosphorus (creatinephosphoric acid). A quantity of the solution expected to contain 0·08 mg. or less of labile phosphorus is taken in a 15 c.c. flask, made up to nearly 10 c.c., and treated with 2 c.c. each of the sulphuric acid and molybdate stock reagents. After 60 minutes, when the creatinephosphoric acid has been hydrolysed, 1 c.c. of the third reagent is added, the solution made up to 15 c.c., stirred, and left for 30 minutes. The precipitate of barium sulphate present is removed by centrifugalization, and the colour is compared with that of a phosphate standard of appropriate strength.

(*b*) A quantity of fraction *A* calculated to contain 0·08 mg. of total phosphorus is taken in a pyrex boiling tube and boiled down with 2 c.c. of the stock sulphuric acid reagent. When all water has been driven off the tube is allowed to cool, one or two drops of Merck's perhydrol are

added and the tube gently heated whilst the decomposing hydrogen peroxide oxidizes the organic matter present. After cooling again water is added and the fluid again boiled down to destroy the excess of hydrogen peroxide and any persulphate which may be present. The contents of the tube are washed with not more than 10 c.c. of water into a graduated cylinder. 2 c.c. of the stock molybdate reagent and 1 c.c. of the hydroquinone reagent are added, the volume is made up to 15 c.c. and measured. After 30 minutes the barium sulphate precipitate is removed by centrifugalization and the solution is estimated colorimetrically.

Fraction B.

(*c*) The orthophosphate is determined in the same manner as the labile phosphate (fraction *A*(*a*)) save that it is unnecessary to wait 60 minutes before adding the hydroquinone reagent.

(*d*) A quantity of solution calculated to contain 0·08 mg. of ortho- and pyrophosphate is treated with 0·5 c.c. of 8*N* HCl and diluted to 4 c.c. with water (the resulting acidity being *N*/1), and the solution is kept in boiling water for 7 minutes. The tube is then chilled and the contents washed into a calibrated measuring cylinder. 1·3 c.c. of the stock sulphuric acid reagent are added so that the total acidity (sulphuric and hydrochloric) is equivalent to 2 c.c. of the stock sulphuric acid reagent, and the solution made up to 12 c.c. The remaining Briggs reagents are added, and the final volume (about 15 c.c.) measured. After standing 30 minutes, the barium sulphate precipitate is removed as before and the solution estimated colorimetrically. This simple technique is made possible by the fact that HCl can replace sulphuric acid in the Briggs solution equivalent for equivalent without any effect on the tint or intensity of the blue colour (Table V).

TABLE V. Effect of replacing sulphuric acid by hydrochloric acid in estimation of phosphorus by Briggs method.

Sulphuric acid	2	1·5	1	0·5	0 c.c.
Hydrochloric acid	0	1·5	3	4·5	6 c.c.
Recovery p.c.	100	100·3	99·8	100·3	100·1

4 c.c. standard phosphate solution (0·8 mg. P) was present in each case. The total acidity in all cases was equivalent to 2 c.c. 5·5*N* sulphuric acid.

(*e*) The total phosphorus in this fraction is estimated exactly as under (*b*).

COMPARISON OF NEW AND OLD METHODS.

The results obtained by the old direct method are usually 1 to 2 p.c. higher than those obtained by the new method on the same acid extract of muscle. The difference may in part be due to the presence of barium

in the fractions estimated in the second method since addition of barium to a trichloroacetic acid extract of muscle without any subsequent fractionation of the salts frequently produces the same very slight lowering of the result. The difference is sufficiently small to be unimportant for most purposes (Table VI).

TABLE VI. Comparison of phosphorus fractions in muscle extracts by the old and new methods.

Experiment	Orthophosphate and phosphagen	Pyrophosphate	Organic phosphate	Total phosphate
1. Old	95	26½	22½	144
New	92½	27½	21½	141½
2. Old	89½	32	26	147½
New	87½	32	26½	146
3. Old	85	32	26	143
New	83½	30	26½	140

The results quoted in the first column for the new method are the sum of the separately obtained values for the orthophosphate and phosphagen, and in the third column the sum of the two organic phosphorus fractions, also separately estimated. Results are given in mg. P per 100 g. of muscle.

RANDOM ERRORS.

In Table VII are reproduced the results of a quadruplicate analysis of a muscle sample. The total acid-soluble phosphorus was determined in five fractions exactly as described in the preceding section. The column "soluble" esters refers to the phosphoric esters with soluble barium salts and is the difference between the total phosphorus and the directly estimable phosphorus (creatinephosphoric acid) in the soluble fraction. The column "insoluble" esters is similarly derived: it is the phosphorus in the insoluble fraction not accounted for as ortho- or pyrophosphate. It will be seen that two-thirds of the values given lie within 0·5 mg. p.c. of the mean. The degree of freedom from random error is satisfactory.

TABLE VII. Four independent analyses of a trichloroacetic acid extract of a partially fatigued muscle.

Exp.	Ortho-phosphate	Phos-phagen	Pyro-phosphate	"Soluble" esters	"Insoluble" esters	Total P
1.	54·7	37·0	28·9	7·9	14·1	142·6
2.	55·0	36·2	28·2	7·9	15·7	143·0
3.	54·4	35·6	29·7	8·4	14·9	143·0
4.	55·3	35·4	29·7	7·9	13·9	142·2
Mean	54·9	36·0	29·1	8·0	14·7	142·7

The actual analyses made in each case were creatinephosphoric acid, and total phosphorus in soluble fraction; orthophosphate, ortho- and pyrophosphate, and total phosphorus in insoluble fraction. The above results have been obtained by differences and are given in mg. of P per 100 g. of muscle.

Some applications of the method.

The most obvious effect of rapidly induced fatigue in isolated muscles of the frog is a disappearance of phosphagen and an increase in the orthophosphate content, but the two changes are not equal in extent. As we have shown previously (Eggleton and Eggleton(9)) the increment in orthophosphate usually accounts for 60 to 80 p.c. of the phosphagen phosphorus which has gone: the remainder of this phosphagen phosphorus appears in the fraction defined by Embden as "lactacidogen" (Eggleton and Eggleton(10)), that is to say the group of phosphoric esters rapidly hydrolysed by the muscle enzymes when the cut-up muscle is incubated in a bicarbonate buffer. To be explicit on this point, some previously published results have been reproduced in Table VIII, from which it will be seen that the amount of organic phosphorus remaining after the incubation was the same for resting and fatigued muscles.

Table VIII. The effect of fatigue on the "lactacidogen" content of muscle.

	Orthophosphate and phosphagen	Organic phosphate	Total phosphate
Killed immediately			
Resting	85	76	161
Fatigued	65	93	158
Incubated			
Resting	116	39½	155½
Fatigued	115½	39	154½

The "non-lactacidogen" phosphoric esters are not altered in amount by fatigue. Results are given in mg. of phosphorus per 100 g. of muscle.

The new method of analysis yields further information on this point. From the results quoted in Table IX it is clear that the ester formed as a result of activity has a soluble barium salt: there is in general no change in the amount of pyrophosphate in the muscle (confirming Lohmann(14)) and only a slight rise, if any, in the amount of phosphoric esters with insoluble barium salts. There is no change in the total quantity of acid-soluble phosphorus. It may be noted in passing that the analytical figures supply what is probably a maximum value for the amount of hexosemonophosphoric ester in the resting muscle, for this ester if present would be found in the fraction with soluble barium salts. The amount is not greater than 5 to 7 mg. of phosphorus, which corresponds to something less than 40 mg. of hexose, per 100 g. of muscle.

The possibility was suggested in an earlier paper that since the phosphagen contents of resting muscles of different types appeared to be directly correlated to their normal ability for rapid energy output,

TABLE IX. The effect of rapidly induced fatigue on skeletal muscles of the frog.

		Total phosphate	Ortho-phosphate	Phos-phagen	Pyro-phosphate	"Soluble" esters	"Insoluble" esters
1.	Resting	142½	24	40	20	5	11
	Fatigued	142½	38	18	23½	12	8½
	Change	—	+14	−22	+ 3½	+ 7	− 2½
2.	Resting	105	20	39	21½	5½	14
	Fatigued	105½	37½	16½	24	10	12
	Change	—	+17½	−22½	+ 2½	+ 4½	− 2
3.	Resting	121½	19½	40	24	4½	12
	Fatigued	120½	40½	5	24	15	15½
	Change	—	+21	−35	0	+10½	+ 3½
4.	Resting	134	18½	49	20½	2	10
	Fatigued	125½	53½	4½	18½	11	12½
	Change	—	+35	−44½	− 2	+ 9	+ 2½
5.	Resting	138½	20½	47½	21	1	10
	Fatigued	138½	34½	26	21	7½	11
	Change	—	+14	−21½	0	+ 6½	+ 1
6.	Resting	112½	15	42½	22	9	11½
	Fatigued	111½	38	12	21½	15½	12
	Change	—	+23	−30½	− ½	+ 6½	+ ½
	Mean change	—	+21	−29½	+ ½	+ 7½	+ ½

The muscle pairs were in most cases the gastrocnemii of the Hungarian (giant) species of *R. temporaria*. The fatigued muscle had been tetanized directly without load for 1–2 minutes. The total phosphate values are given in mg. of phosphorus per 100 g. of muscle; the five fractions are expressed as percentages of the total.

the function of phosphagen might be that of an accelerator of muscular activity. The recent work of Nachmannsohn(11) has brought out clearly the existence of such a relationship, and it seemed possible that the phosphagen of a muscle might not be distributed evenly throughout its bulk, but might be absent from any part of the muscle which contained no nerves or motor end plates. Accordingly the distal ends of the sartorii and graciles (which are known to contain few if any nerve endings) of some giant frogs were cut off and estimated separately from the main mass of muscle. No difference was detectable however, either in the total phosphorus content or in the distribution of the five phosphorus fractions determined (Table X).

TABLE X. Comparison of muscle tissue free from motor end plates with remainder of muscle.

	Ortho-phosphate	Phos-phagen	Pyro-phosphate	"Soluble" esters	"Insoluble" esters	Total phosphate
Nerve-free	31·8	55·4	25·7	5·8	15·6	134·3
Remainder	32·2	55·4	25·4	5·3	16·1	134·4

The lower portions (about one-tenth) of the sartorii and graciles of several frogs were analysed separately from the main bulk of the muscle. The results are given in mg. of phosphorus per 100 g. of muscle.

Clearly the phosphagen of the muscle is associated with the fibres themselves and not with the nerve endings.

The slow death of an isolated muscle through oxygen lack is marked by a steady formation of orthophosphate, a process finishing only when some 90 p.c. of the muscle phosphorus has become inorganic. During the earlier part of the process the only phosphoric ester which can be shown to be irreversibly destroyed is phosphagen. This we demonstrated in the case of the coraco-mandibular muscle of the ray (1928). In the experiments then made a single muscle was cut into several pieces and kept in hydrogen, analyses being made by the old direct method. We have repeated the experiments, using in each experiment a number of uninjured frog muscles and performing the analyses by the new method. The results (Table XI) confirm the earlier ones and bring to light further interesting relationships which the older method failed to reveal. For 24 hours after the beginning of anærobiosis phosphagen steadily disappears and its phosphorus appears quantitatively as orthophosphate. The sum of the two does not change. Later, when almost all the phosphagen has gone, a breakdown of pyrophosphate begins, and continues till no pyrophosphate is left. Later still, the phosphoric esters with insoluble barium salts are attacked and ultimately are completely destroyed. But during the whole period of 50 to 60 hours there is no sign of a disappearance of the acid-stable group of esters with soluble barium salts: indeed they show a steady tendency to increase in amount throughout the whole time. The amount is more than doubled. It is not

TABLE XI. The effect of prolonged oxygen lack on the distribution of phosphorus in the skeletal muscles of the frog.

Time in hours	Muscle	Total	Ortho-phosphate	Phos-phagen	Pyro-phosphate	"Soluble" esters	"In-soluble" esters
0	1 Gr. L.	128·8	13·1	53·3	21·6	2·6	9·6
0	1 S. R.	128·0	15·1	49·4	28·5	4·3	2·7
4	1 G. L.	129·2	31·8	30·6	21·4	5·7	10·5
4	2 Gr. R.	135·1	28·2	36·3	24·0	5·5	5·8
19	2 S. L.	141·1	47·8	12·2	22·4	8·4	9·4
19	2 G. R.	148·4	46·2	14·2	22·5	6·6	10·6
26	1 Gr. R.	141·9	51·5	9·2	19·0	9·1	11·3
26	1 G. R.	129·5	62·6	6·2	14·4	7·3	9·4
43½	2 G. L.	133·0	65·1	3·0	13·4	7·5	10·9
43½	1 S. L.	136·2	83·7	0	2·9	8·9	4·4
50½	2 S. R.	144·0	90·6	0	2·8	9·4	0
50½	2 Gr. L.	133·0	89·7	0	0	10·3	0

The gastrocnemii (G.), graciles (Gr.) and semimembranosi (S). from the left (L.) and right (R.) legs of two frogs (1 and 2) were kept in moist hydrogen at room temperature for the stated time. The total acid-soluble phosphorus is given in mg. per 100 g. of muscle; the five fractions are expressed as a percentage of this figure. The frogs used were the giant species of *R. temporaria* from Hungary.

possible, nor fortunately is it necessary, to offer an "explanation" of these results. They are introduced here primarily as an illustration of the use of the new method of analysis.

A COMPARISON OF DIFFERENT TISSUES.

The technique outlined in this paper is applicable without modification to plain muscle, blood, and several other tissues, but cannot be applied to liver owing to the presence of some pigment which interferes with the estimation, and which we have not succeeded in removing by any simple treatment. The results that have been obtained illustrate the particular value of the method in detecting traces of phosphagen (Table XII).

TABLE XII. Phosphorus distribution in certain organs of some vertebrates.

Tissue	Ortho-phosphate	Phos-phagen	Pyro-phosphate	"Insoluble" esters	"Soluble" esters	Total P
Heart						
Guinea-pig	27·5	5·2	24·3	18·9	16·2	92·1
Stomach						
Rabbit	32·0	2·3	14·5	11·6	17·7	78·1
Rabbit	24·7	5·1	19·7	14·5	12·2	76·2
Frog	18·5	0·5	8·5	8·0	17·0	52·5
Uterus						
Rabbit	11·6	1·4	8·4	8·8	22·6	52·8
Guinea-pig	14·0	Trace	7·8	11·2	17·0	50·0
Testis						
Rat	10·7	0·9	11·0	8·7	42·7	74·0
Rat	11·7	0·7	12·9	11·8	40·0	77·1
Cat	9·1	0·6	5·3	5·0	29·2	49·2
Rabbit	10·6	2·0	5·6	7·6	32·8	58·6
Guinea-pig	7·6	2·6	17·0	13·0	37·2	77·4
Kidney						
Rabbit	18·0	0	10·3	9·6	50·3	88·2
Cat	10·1	0	8·3	6·4	36·0	60·8
Spleen						
Rabbit	13·7	0	13·2	17·8	42·8	87·5
Cat	9·8	0	10·9	7·9	29·1	57·7
Blood						
Dog	6·0	0	2·0	17·5	1·0	26·5
Human	3·0	0	3·8	12·7	1·0	20·5
Rabbit	5·1	0	(... 19·7 ...)		3·5	28·3
Cat	4·3	0	1·2	—	1·4	—

The figures in columns 4 and 5 refer to esters having insoluble and soluble barium salts respectively. All values are given in mg. of P per 100 g. of tissue (100 c.c. in the case of blood).

Analyses of blood from four different vertebrates reveal no trace of phosphagen. The fraction in which phosphagen would appear is completely blank. We cannot confirm the statement of Fiske and Subbarow(12) that the Briggs reagents develop a blue colour in the absence of phosphate. If clean glassware and pure reagents are used a "blank" test develops no blue colour. There is a small trace of pyrophosphate in blood according to our figures, and an even smaller amount of organic phosphate with a soluble barium salt—about 1 mg. per 100 c.c. Most of the blood phosphorus is in the form of esters with insoluble barium salts.

The spleen and the kidney of the rabbit and cat we have also found to contain no phosphagen, though according to Ferdmann and Feinschmidt(13) appreciable quantities of phosphagen can be found in the spleen. These two organs are alike in their content of the different phosphorus fractions, and are remarkable for the proportion and absolute amount of "soluble" esters: more than half the phosphorus present is in this group.

The uterus and stomach have been taken as examples of unstriated muscle. Both contain labile phosphorus in small amounts. We have previously reported failure to detect phosphagen in the stomach muscle of the frog when using the old technique. The new method reveals a trace of labile phosphorus in this organ such as certainly could not have been detected by the old method. In the thick muscular coat of the pyloric end of the stomach of the rabbit larger amounts are found, though the quantity is always less than in any striated muscle we have examined. Except for a rather high proportion of "soluble" phosphoric esters there is nothing remarkable in the rest of the analytical results of these organs.

In all cases where a tissue apparently contains a minute amount of labile phosphorus it is our custom to boil a separate sample of the original trichloroacetic acid extract for 2–3 minutes, a treatment which destroys any phosphagen present, and repeat the analysis for phosphagen. In all cases this has been found to give a clear negative result.

The most interesting results quoted in Table XII are the analyses of the testes of rabbit, guinea-pig, cat and rat. These organs all contained labile phosphorus in an amount comparable to the stomach muscle. The testes are said to contain very few muscle fibres: it is highly improbable that these few fibres could account for the presence of this "labile" phosphorus assuming that it represents phosphagen. It seems possible that this "labile" phosphorus is really a constituent of the spermatozoa.

Pyrophosphate.

Since this paper was written we have had the privilege of reading the manuscript of a paper by Dr Lohmann in which he states that the pyrophosphate of muscle exists in combination with adenylic acid in a molecule which therefore contains three phosphorus atoms. Two of these (linked as pyrophosphate) are easily hydrolysed by acid, whilst the third is an orthophosphate radicle relatively resistant to hydrolysis. Reference to Tables IV, VII and IX in this paper reveals two interesting facts which seem to be associated with this discovery. In the first place the amount of the "insoluble organic phosphate" in a voluntary muscle is almost exactly one-half of the amount of pyrophosphate. If we make the assumption (for which at present we have no direct evidence) that the adenylic acid phosphorus associated with the pyrophosphate is estimated in this fraction we can calculate the amount of phosphorus in this fraction in excess of that present as the pyrophosphoric-adenylic acid complex. It amounts to practically nothing. In the case of the twelve muscles in Table IX the "excess" is 1, − 3, 3, 0, 0, 3½, − ½, 3, − ½, ½, ½, and 1 p.c. of the total acid-soluble phosphorus. This suggests strongly that the fractions which we have called "pyrophosphate" and "insoluble esters" respectively measure together the pyrophosphate-adenylic acid complex of Lohmann. If this be so there remains practically none of the acid-soluble phosphorus of the muscle to be accounted for, since the group we have called "soluble esters" probably consists in the main of hexosemonophosphate.

As regards other tissues it will be seen in Table XII that the "insoluble ester" fraction is never less than half the pyrophosphate fraction. Indeed, the two fractions are usually about equal in amount. This suggests the presence of some compound in the "insoluble ester" fraction which is absent from voluntary muscle.

Summary.

1. The suggestion is made that the creatinephosphoric acid which can be isolated from voluntary muscles may not be identical with phosphagen. The latter may contain in addition some component of a colloidal nature.

2. Mention is made of some improvements which make the previously published method for the estimation of phosphagen more convenient and useful over a wider range. Resting frog muscles which contain 12–18 mg. of inorganic phosphorus and 55–70 mg. of phosphagen

phosphorus per 100 g. of muscle may be conveniently analysed by this improved technique.

3. A method is described for the separation and separate estimation of the inorganic (ortho)phosphate and phosphagen in 0·5 to 1 g. of muscle, based upon the different solubilities of the barium salts of orthophosphoric acid and creatinephosphoric acid. The method is primarily intended for use where the phosphagen content of the tissue is very low in comparison with the inorganic phosphate content; by it one can detect with certainty one part of phosphagen phosphorus in 200,000 parts of tissue, irrespective of the inorganic phosphate content. The use of the method need not, however, be restricted to such cases.

4. An easy extension of the technique permits of the additional estimation of pyrophosphate and of two distinct fractions of phosphoric esters, with soluble and insoluble barium salts respectively.

5. Analyses of resting voluntary muscles by the above method give the distribution of acid-soluble phosphorus as follows: orthophosphate 15 p.c., pyrophosphate 20 p.c., phosphagen 50 p.c., acid-stable phosphoric esters with soluble barium salts ("soluble" esters) 4 p.c., with insoluble barium salts ("insoluble" esters) 11 p.c.

6. Fatigue increases the orthophosphate content and the amount of "soluble" esters, diminishing the phosphagen content, but leaving unchanged the pyrophosphate content and the content of "insoluble" esters.

7. The lower end of the sartorius muscle of the frog, which contains no nerves or motor end plates does not differ from the remainder of the muscle in its analysis by the above method.

8. Prolonged oxygen lack produces no change in frog muscles in respect of the above fractions (other than the breakdown of phosphagen and the formation of orthophosphate), until almost all the phosphagen has disappeared. At this stage pyrophosphate and the "insoluble" esters begin to disappear, and after 50 to 60 hours of anærobiosis the muscle contains no phosphagen, pyrophosphate, or "insoluble" esters, though the quantity of "soluble" esters has, if anything, increased and amounts to some 10 p.c. of the acid-soluble phosphorus.

9. Blood from four different vertebrates when analysed by the new technique was found to contain no phosphagen; phosphagen was also found to be absent from the spleen and kidney of the rabbit and cat. The uterus of the rabbit and guinea-pig, and the stomach of the rabbit and frog were found to contain labile phosphorus in amount varying between a trace and 5 mg. per 100 g. The testes of the rabbit, guinea-pig,

cat and rat contain labile phosphorus, but it is uncertain whether this represents phosphagen and whether it is derived from the supporting tissue of the testis or from the spermatozoa.

10. It is suggested that there may be a close relationship between the amount of pyrophosphate in a voluntary muscle and the amount of orthophosphoric esters which have insoluble barium salts.

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THE PHYSIOLOGICAL ACTIVITY OF ADENINE COMPOUNDS[1] WITH ESPECIAL REFERENCE TO THEIR ACTION UPON THE MAMMALIAN HEART.

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ERRATUM

Description of Fig. 5. Page 171.
for 0·55 c.c. "Pitressin" *read* 0·05 c.c. "Pitressin"

animal so that the pre-injection state was completely restored. It seemed possible by using this transient disturbance of the heart beat as a biological test to isolate the responsible substance, and by enquiring into its chemical nature to obtain some idea of one of the chemical factors involved in cell function not only in the heart but in the body generally.

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Different fundamental properties of the cell are called into play in the production of a normal heart beat, and any change in these qualities is immediately reflected in the character of the beat. The heart, therefore, is an admirable test object for any substance which may be supposed to influence cell activity, and if the influence is such that an abnormal rhythm results, it can be studied electrocardiographically with ease in the intact animal. Moreover, if the substance is neither cumulative nor toxic the duration of the abnormal rhythm produced will give a quick and accurate assay of the amount of substance present in successive samples.

We had found that simple extracts of heart muscle[4] and other tissues when injected intravenously into the whole animal disturbed the cardiac rhythm in a constant and definite manner, and the substance or substances involved were dealt with quickly and efficiently by the whole animal so that the pre-injection state was completely restored. It seemed possible by using this transient disturbance of the heart beat as a biological test to isolate the responsible substance, and by enquiring into its chemical nature to obtain some idea of one of the chemical factors involved in cell function not only in the heart but in the body generally.

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substance; in the second part we shall deal with its physiological activity. This has, for the most part, been concerned with its action upon the heart, but we would emphasize at the outset, though we feel this will be apparent from our observations, that we do not consider that the substance acts specifically upon the heart but is concerned with cell activity in general.

Part I.

Isolation of the substance.

If freshly minced bullock's heart muscle is extracted with 5 p.c. trichloracetic acid in the cold, and the precipitated proteins filtered off, this filtrate has a definite action upon the heart. The filtrate from 100 g. of muscle when injected intravenously into a dog produces a transient sinus bradycardia. This reaction, without undergoing any obvious change, can be repeatedly obtained in the same animal by reinjection. Complete atropinization of the animal does not abolish this reaction, so that it cannot be due to stimulation of the vagus nerve, which it so closely resembles. It is not due to inorganic constituents, as incineration or boiling with acid completely destroys the action of the extract.

For purposes of isolation a quick and accurate measure of the amount of constituent present in any injection must be available. From the practical point of view the duration of the sinus bradycardia in the dog is of little value as a relatively large amount of the extract is required for each injection, and the onset and offset of the reaction are only to be determined by detailed measurement. Several animals have been investigated to find a more suitable effect, and the guinea-pig has been found to react in such a manner that the duration of the effect produced by an extract from 0·5 g. of muscle can be accurately and quickly measured.

The effect in the guinea-pig. If the simple extract from 0·5 of a g. of heart muscle is injected into the jugular vein of an anæsthetized guinea-pig the auricular rate is but little disturbed and a transient high grade heart block develops (Fig. 1). The heart beat is recorded electrocardiographically from two needles inserted beneath the skin of the right fore leg and left hing leg respectively. Analysis of the records thus obtained shows that about 3 seconds after the beginning of the injection heart block abruptly develops, which is initially complete but which after about 6 seconds passes gradually off through the stages of 3 : 1, 2 : 1 block, 1 : 1 response with prolonged P–R intervals; the preinjection condition being reached in 9–12 seconds. The auricular rate in such a

record is slightly retarded. This reaction can be repeatedly obtained by reinjection, and remains constant in type and duration. As a measure of the amount of responsible constituent present in any injection we have used the time elapsing between the onset of the complete block and the reappearance of the first 1 : 1 response, as no material error is thus introduced. The abrupt onset of the block and the abrupt return of the 1 : 1 response are so obvious in the string movements that often it is not necessary to take a record, the duration being measured by a stop watch[1]. If larger injections are employed, the auricular rate is definitely slowed: this confuses the record, as the 1 : 1 responses return at a low rate, and simulate a 2 : 1 response; with smaller injections the decreased total duration makes comparison difficult. We have found that a reaction of some 10 to 12 seconds' duration and an injection of $\frac{1}{4}$–1 c.c. of extract to be the most suitable, and have either concentrated or diluted our extracts to obtain this reaction. We had noted in some animals that even with weak injections the auricular rate is definitely retarded and that full atropinization rendered this slowing much less definite, and in some cases actually shortened the duration of the heart block. To obviate this effect, 0·1 of a c.c. of 1 p.c. solution atropine sulphate has been injected as a routine measure throughout. The temper-

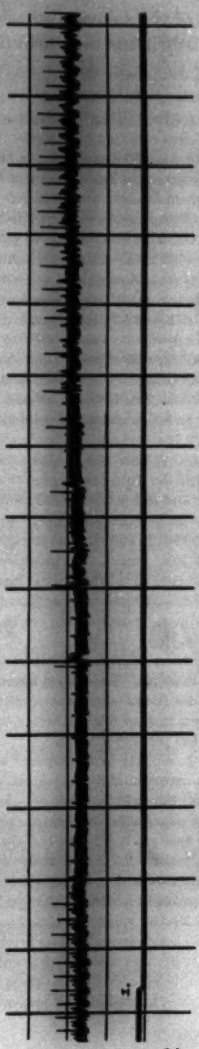

Fig. 1. Guinea-pig. Electrocardiographic record (1*A* n). Influence of intravenous injection of 1 c.c. of extract from heart muscle (0·5 g.). Injection commenced 3 secs. before and terminated at I. Time marker = 1 sec.

[1] It is quite possible to measure the duration of the heart block without any instrumental means. The sudden onset and equally sudden offset are easily appreciated by placing the forefinger and thumb upon the thorax.

ature of the animal also affects the duration of the reaction, and consequently it has been maintained constant throughout the observations. Using this method we have been able to test the distribution of the responsible substance after various chemical procedures and have isolated a crystalline product on the following lines.

Extraction. The extraction is best carried out in an acid medium and at a moderately high temperature, but if the extracting agents are made sufficiently acid, a good yield can be obtained at room temperature.

Many reagents have been tried to extract the substance, and trichloracetic acid has proved the most suitable. It is used as a 5 p.c. watery solution. The finely minced tissue is suspended in this solution (1 c.c. of 5 p.c. trichloracetic acid being used for every gramme of tissue) and allowed to stand over night, the fluid being then pressed out and filtered. When larger quantities of tissue are used, 1 p.c. trichloracetic acid is used. The tissue is suspended in the solution (1 c.c. of 1 p.c. trichloracetic acid being used for every gramme of tissue) and with occasional stirring is allowed to stand for half an hour, it is then heated up to 90° C., and subsequently cooled. The fluid is pressed out and filtered. The filtered fluid from both processes is neutralized at this stage and tested by injection into a guinea-pig.

Isolation. The isolation of the substance depends upon the insolubility of its lead, copper, and zinc salts, the solubility of its barium salt in water, and its insolubility in alcohol.

To the filtered and cooled extract a hot saturated solution of barium hydroxide is added till the fluid just begins to colour phenolphthalein. A precipitate forms which is inactive and this is filtered off. Solid neutral lead acetate is added to the filtrate sufficient to make its concentration up to 4 p.c. After this has dissolved, 1 p.c. of strong ammonia (s.w. = 0·880) is added. The precipitate which contains the active substance is separated on a Büchner funnel. This precipitate is suspended in a little water and 20 p.c. sulphuric acid is stirred in till the fluid is just acid to thymol blue. The lead sulphate is filtered off on a Büchner funnel, and the filtrate, whose volume should be about one-tenth of the original volume, is made alkaline with strong caustic soda till it just begins to colour phenolphthalein.

Crystalline copper sulphate is now added in sufficient quantity to bring its concentration up to 20 p.c. The fluid is boiled up rapidly, cooled and the precipitate separated upon a Büchner funnel and washed with water. This precipitate, which contains the active substance, is suspended in water the volume of which should be about one-twentieth of that of the primary extract.

The precipitate is decomposed with H_2S, the copper sulphide filtered off and washed, and the excess of H_2S aerated off. A hot saturated solution of barium hydroxide is now added till the fluid just colours phenolphthalein and the precipitate filtered off. To the filtrate a strong solution of zinc acetate is now added until no further precipitate is formed, and the solution is brought quickly to the boiling point and then cooled down. The precipitate, which contains the active substance, is collected on a Büchner funnel and washed with water. It is then suspended in a very small volume of water and solid trichloracetic acid is gradually added till the precipitate is completely dissolved. Water is added to this solution till the volume is about one-thirtieth of the primary trichloracetic acid extract and H_2S passed through till no further precipitate is formed. The zinc sulphide precipitate is filtered off, and washed with water. H_2S is again passed through the filtrate, sodium carbonate being added at this stage till the reaction is neutral to litmus. The zinc sulphide precipitate is filtered off and washed with water.

The filtrate is now acidified with acetic acid till the reaction is distinctly acid to litmus. It is then concentrated at low pressure and temperature (below 40° C.) to a small volume (about one-hundredth of the volume of the primary extract), and made alkaline to phenolphthalein by the addition of a hot saturated solution of baryta. Four times its own volume of alcohol is now added, and the precipitate which contains the active substance collected in a Büchner funnel. The precipitate is suspended in a small volume of cold water and filtered; the residue on the filter is suspended in a small volume of warm water and filtered. The final residue is extracted with a small volume of boiling water and filtered. All these three filtrates are united, and silver nitrate is added till no further precipitate is formed. The precipitate is collected in a Büchner funnel and washed with water. It is then suspended in as small a volume of water as possible and a strong solution of hydrochloric acid added drop by drop till the fluid is just acid to thymol blue. The fluid is filtered off or centrifuged. To the clear filtrate an equal volume of alcohol and three times its own volume of acetone is added, when the substance separates out in clusters of small crystals. An equal amount of ether is now added and the fluid placed in an ice-chest over night. The crystals which separate out are collected on a filter, dissolved in as small a volume of hot water as possible and H_2S passed through to remove the remaining traces of silver. The hot solution is filtered through a filter covered with fine asbestos, and alcohol added to promote crystallization. The solution is placed in the ice-chest and the substance separates out in beautiful long needle crystals. These are separated the next day, washed with 50 p.c. alcohol, acetone, ether, and then dried. From the mother liquor additional crystalline substance can be separated by adding further quantities of alcohol, acetone and ether.

The crystals thus obtained show no change of activity upon recrystallization.

The chemical nature of the active substance.

The crystalline substance is quite stable in neutral solution but is rapidly hydrolysed by mineral acids. On qualitative analysis it gives carbon, hydrogen, nitrogen, phosphorus and oxygen (indirect). It gives a strong Kossel test indicating the presence of an alloxuric base. The substance gives no reactions for guanidine. After hydrolysis a heavy crystalline precipitate is formed with picric acid. After recrystallization these crystals melt and decompose at 280° C., being thus identified as adenine picrate. The substance gives no reaction with α napthol but a strong and typical reaction with orcinol, showing the presence of a pentose. After boiling with strong nitric acid the substance gives a typical precipitate with molybdate.

These experiments show that three of the constituents are adenine, a pentose, and phosphoric acid respectively, which suggests that the substance is identical with or closely related to adenylic acid. The work of Bass(1) had suggested that this substance is present in animal tissues, while Hoffman(2) has isolated it from blood, and Pohle(3) from brain. Embden and his collaborators Zimmerman and Schmidt have isolated it from striated muscle and thoroughly studied this substance (4, 5, 6). The formula of this substance is $C_{10}H_{14}O_7N_5P - H_2O$ (molecular weight = 365·19 g.). The elementary analysis of our substance suggests

a formula identical with or in close agreement with that of adenylic acid: viz.

	C	H	N
Percentage calculated for adenylic acid	32·9	4·4	19·2
Percentage found*	32·7	3·9	19·0
	32·41	4·2	18·17†

* Nitrogen by method of Dumas. Both analyses were made by Dr A. Schoeller, Berlin.

† The substance, after drying *in vacuo* at 60° C., gave 19·88 p.c. nitrogen (Kjeldahl estimation).

Our recrystallized substance melts and decomposes between 194° and 200° C. The melting point is not sharp as the substance begins to decompose just above 190° C., and the slower it is heated up the more decomposition products are formed, with a consequent lower melting point. Embden and Schmidt give a melting point of 194°–200° C. for muscle adenylic acid. When dissolved in 2 p.c. NaOH our substance gives $a_D^{21} = -44$, while Embden and Schmidt find for muscle adenylic acid $a_D^{21} = -47{\cdot}5$. If their estimation is of anhydrous material, the agreement is quite close. Our substance has strong acidic properties; 2 mg. is neutralized by 0·99 c.c. of 0·011 *N* NaOH, phenolphthalein being used as indicator. This corresponds to an equivalent weight of 184 and a molecular weight of (184) *n* ((184) 2 = 368).

Prof. Embden has kindly supplied us with his muscle adenylic acid and we have been able to make some further comparative observations. The mixed melting point of adenylic acid and of our substance is identical with that of either substance alone. The brucine salts prepared from both substances behave identically upon crystallization, both becoming transparent at 168° C. and melting at 188° C. We are strongly of the opinion, as a result of these observations, that our substance is identical with adenylic acid[1], and we shall refer in this paper to the substance isolated by us as adenylic acid.

The activity of related compounds[2].

It has been found that the hydrolysate from yeast nucleic acid hydrolysed at neutral reaction at 145° C. has an activity which is similar

[1] The residue left after combustion (metaphosphoric acid) had suggested that our substance had a bigger molecule with a pyrimidine base. We could however obtain no further evidence of this base and the small quantity of residue is probably to be accounted for by a loss of ash, as no special precautions had been taken to prevent such a loss.

[2] Through the kindness of C. H. Fiske we have been able to test the action of the pyrophosphoric or diphosphoric ester of adenylic acid. Its action is similar to that of adenylic acid and adenosine.

in every way to that of adenylic acid. The substance in the hydrolysate responsible for the activity has been isolated in crystalline form and has been identified by its melting point and the products formed by splitting it as adenosine. A number of closely related nucleic acid derivatives have been prepared and tested upon the guinea-pig. The following have been found to be inactive: yeast nucleic acid, thymo-nucleic acid, yeast adenylic acid[1], B. nucleoproteid, guanylic acid, guanosine, guanine, inosinic acid[1] and adenine. It can also be postulated that the pyrimidine nucleotides and nucleosides present in hydrolysates of yeast nucleic acid prepared at neutral reaction at 115° C. and 145° C. respectively are inactive. We find, moreover, that the activity does not lie in the imidazol formation present in the adenine molecule, as 4 (5)-amino-methyl-glyoxaline[2] is inactive.

The chemical nature of the action of adenylic acid and adenosine.

The delayed action of adenylic acid and adenosine suggests that these substances undergo a chemical change before their activity is seen; they do not suddenly disturb the physico-chemical state of the cell. The chemical nature of the substances also supports this view, for it is surprising that, belonging as they do to the nucleic acid group, they have such a high activity. The whole activity of the substances can be abolished by splitting off the inert carbohydrate group (adenine) by withdrawing the amino group (inosinic acid) by placing it in a different position (guanosine). Even if the phosphoric acid linkage is changed (yeast adenylic acid) the activity is lost. It has been shown by Embden and his collaborators that adenylic acid under certain conditions is rapidly deaminated by muscle tissue, and Schmidt(5) has made a comparative study of a number of nucleic acid derivatives with regard to the ease with which this deamination occurs. He has found that while muscle adenylic acid and adenosine are easily deaminated, yeast adenylic acid, adenine, guanine, guanosine and guanylic acid are not deaminated at all or only very slowly. We have found that the ease of deamination and the activity of the substance runs so parallel that it is only reasonable to conclude that the two, adenylic acid and adenine, owe their activity to the ease with which they are deaminated in the body. By this deamination ammonia is formed. The question must come into consideration as to whether the biological activity is associated with this alkalinity.

[1] We are indebted to Prof. Embden for this preparation.

[2] We are indebted to Boots Pure Drug Co., Nottingham, for this preparation.

Distribution of adenylic acid.

Adenylic acid is not a specific constituent of heart muscle. It has been found in blood (1, 7) isolated by Embden and his collaborators from skeletal muscle (4) and by Pohle from brain (3). Judged by the activity of simple extracts of different tissues upon the guinea-pig's heart the substance is a general cell constituent. It appears to be present in approximately the same concentration in heart muscle, skeletal muscle, kidney and brain but in less concentration in the spleen[1]. Its concentration in yeast cells appears to be double that in animal tissues. At the moment we are unable to state definitely whether the activity of the substance is enhanced or suppressed by the other substances present in the crude extracts or whether the absolute amount of the substance present in such extracts can be calculated directly from the intensity of the cardiac change. All our observations, however, indicate that the cardiac change depends only on the amount of the substance present in the extracts, for we have experienced no sudden change or fall in activity during the process of fractionation, and about one-third of the substance as calculated from the primary extract is isolated. Moreover the addition of inorganic salts in quantities in which they naturally occur has no effect upon the activity of the crystalline product. On this assumption, a comparison of the activity of the primary extracts with the activity of the crystalline substances shows that in every 100 g. of heart muscle some 50 mg. of the active substance are present.

After death the substance disappears slowly. An extract of tissue which has stood for 16 hours at room temperature has lost half, while the extract of tissue which has stood for the same time at 37° C. has lost two-thirds of its activity.

Part II.

Physiological activity.

As adenylic acid had been found to be a constituent of many and different types of animal cells, an investigation into its general physiological properties in addition to its detailed action upon the mammalian heart seemed to us very desirable. The finding that adenosine had similar activities gave us the opportunity of such an investigation, for the latter substance could be prepared easily and in large quantities, while the former was only to be obtained with difficulty. We have, therefore, made a series of observations upon the physiological activity of

[1] The organs were obtained from bullocks and sheep.

adenosine, and have made occasional experiments throughout in which the adenosine has been replaced by our adenylic acid. As far as our experiments go, we have been unable to detect any difference in the quality of action of the two substances, and we feel content that the same changes are brought about by adenosine as by adenylic acid and *vice versa*[1].

Quantitative tests show that 0·2 mg. of the substance isolated by us produces a heart block of 12 seconds' duration; this can be duplicated by 0·2 mg. of muscle adenylic acid. Adenosine on the other hand is about 1½ times stronger. This is explained by the smaller size of the molecule of adenosine. This quantitative difference must be taken into consideration if the method described is used to estimate the actual amount of adenylic acid present in any fraction; for, owing to the individual differences in the animals used, the duration of heart block to a known amount of adenosine must be used as a standard. The method, from our observations, seems to offer a reasonably accurate means of determining the actual amount of adenylic acid in different animal tissues.

Observations upon the mammalian heart.

The sinus node and junctional tissue. We have already shown that in the guinea-pig adenosine and adenylic acid, while slowing in slight degree the sinus rhythm, act more strongly upon the junctional tissues so that a high grade heart block is produced. In the other animals we have tested this relation does not hold. In the cat, for instance, a very transient sinus bradycardia is seen after an intravenous injection of 50 mg., while in the rabbit a similar bradycardia occurs only after an intravenous injection of 100 mg. or more. In the dog a well-marked sinus bradycardia develops and this has been studied in detail. In these experiments and in others upon the dog's heart the animal has been anæsthetized with morphine and chloralose and the heart beat electrically registered by a string galvanometer (lead II). The adenosine or adenylic acid has been injected into the femoral vein dissolved in 1 c.c. of saline, the dose[2] being washed by saline solution. The sinus rhythm is consistently slowed by the injection, its rate being reduced by approximately

[1] At our request the British Drug Houses have taken up the manufacture of adenosine.

[2] We have employed in the dog doses up to 100 mg. only, such doses produce very definite effects and the use of higher than these seemed undesirable. The weight of the dogs used varied from 6–8 kilos.

one-half by 20 mg. of adenosine and by two-thirds by 50 mg. The bradycardia does not develop until 10–15 seconds after the injection, and gradually passes away, the preinjection rate being reached about 60 seconds later. Two typical experiments are tabulated in Table I.

TABLE I. Effect of adenylic acid and adenosine upon heart rate of dog. Injection into femoral vein.

		Length of cycle in seconds							
			After injection						
Dog	Dose mg.	Before injection	5″	10″	20″	30″	40″	50″	60″	70″
ND.	20 adenosine	0·55	0·55	1·25	0·83	0·76	0·62	0·62	0·58	0·55
ND.	20 adenylic acid	0·55	0·55	1·11	1·11	0·83	0·67	0·67	0·58	0·55
	0·6 c.c. of 1 p.c. solution of atropine sulphate injected; vagi inactive									
ND.	20 adenosine	0·52	0·50	1·00	1·11	1·00	0·72	0·62	0·58	—
ND.	20 adenylic acid	0·52	0·54	1·00	0·83	0·77	0·77	0·63	0·58	—
PD.	20 adenosine	0·41	0·41	0·57	0·88	0·42	0·39	0·39	—	—
PD.	40 adenosine	0·44	0·44	0·65	1·00	0·82	0·63	0·53	0·48	—
	0·5 c.c. of 1 p.c. solution of atropine sulphate injected; vagi inactive									
PD.	40 adenosine	0·41	0·41	1·15	0·93	0·70	0·54	0·49	0·44	—

An identical effect is obtained after full atropinization (1 c.c. of 1 p.c. solution of atropine sulphate for a dog of 10 kg.), showing that the effect is not due to vagal stimulation. Even during the period of maximum slowing, there is no change in the contour of the "P" wave in the electrical record[1], the position of the "pace-maker" remaining unchanged. In the cat, in addition to the primary bradycardia which occurs within 20 seconds of the injection and which rapidly passes off, a secondary bradycardia sets in at about the 50th second after the injection of either adenosine or adenylic acid and persists for about 40 seconds. The electrical records show that this is also a sinus bradycardia. It differs from the primary bradycardia in that it is vagal in origin, being completely abolished by complete atropinization of the animal (Table II).

TABLE II. Delayed effect of adenosine upon heart rate of cat. Injection into femoral vein.

	Length of cycle in seconds						
	After injection						
Dose mg.	Before injection	40″	50″	60″	70″	80″	90″	100″
50	0·33	0·30	0·29	0·36	0·40	0·41	0·36	0·34
0·4 c.c. of 1 p.c. solution of atropine sulphate injected into femoral vein, vagi inactive								
12	0·28	0·25	0·26	0·28	0·28	0·29	0·29	0·29
50	0·30	0·25	0·24	0·24	0·23	0·24	0·25	—

[1] This is not invariable, a change in the contour of the "P" wave is very occasionally seen, even after full atropinization.

The influence of these substances upon the junctional tissue between auricle and ventricle which is so clearly displayed in the guinea-pig's heart cannot be easily appreciated in the dog or cat owing to the high grade of sinus bradycardia which occurs in these two animals. The slow heart rate affords the junctional tissues a long recovery period, and consequently the impulses travelling from auricle to ventricle are not blocked. On the other hand careful measurement of the electrical record shows that during the period of maximum slowing of the heart the P–R intervals are consistently lengthened, though in very slight degree. The action has been further examined by maintaining the auricular rate constant by applying rhythmic break shocks to the auricle, and, in these circumstances, varying degrees of heart block are constantly obtained. Representative examples of such experiments are tabulated in Table III.

TABLE III. Influence of adenylic acid and adenosine upon the P–R interval of dog's heart. Injection into femoral vein.

		Heart rate per minute and P–R interval in seconds					
	Dose	Before		10–15 secs. after		60 secs. after	
Dog	mg.						
ND	20 adenosine	108	0·090	48	0·109	108	0·087
ND*	20 adenylic acid	114	0·087	60	0·106	102	0·090
PD*	40 adenosine	144	0·107	87	0·115	139	0·105
ND*	20 adenosine	234	0·095	234	0·135		—
ND*	20 adenosine	234	0·103	234	0·134		—
TD*	50 adenosine	220	0·080	220	0·110	220	0·082
UD*	50 adenosine	250	0·082	250	0·111	250	0·084
UD*	50 adenosine	400	0·123	400	2:1		—
VD*	50 adenosine	240	0·102	240	Occasional dropped beats	240	0·130
GAD*	50 adenylic acid	220	0·105	220	0·140		—

* Animal fully atropinized.

From these experiments it is evident that both the substances tested act in a similar manner upon the sinus and the junctional tissues respectively. The rate at which the normal impulse is formed in the sinus is definitely retarded and conduction in the junctional tissues is impaired so that impulses quickly following upon one another pass through with difficulty. In the guinea-pig the sinus rhythm is affected but little, and high grade heart block is seen. In the dog and cat the sinus rhythm is so considerably retarded that the action upon the junctional tissues cannot be appreciated on rough examination. We can offer no explanation for the difference in the reaction of the animals tested.

The auricular muscle. Experiments have been made to test the influence of adenosine upon the absolute refractory period of the auricular muscle in the dog's heart. The rate of auricular beating has been

maintained constant by means of rhythmic break shocks passed through electrodes embedded in the muscle. The refractory period has been tested by passing an extra shock, using the apparatus described by one of us (8), through the same pair of electrodes. The rhythmic and testing shocks and the responses of the auricle have been recorded electrically and simultaneously by the method originally described (9). The refractory period is thus tested at the point of rhythmic stimulation, and to eliminate the effects of the local stimulation of vagal fibres by the rhythmic and testing shocks, the animals have been fully atropinized. The strength of the testing shocks has been about 10 times the threshold value. The maximum activity of adenosine, as judged by its action upon the sinus node, is about 15–30 seconds after the injection, and the refractory period has been tested during this period; a second estimation being made about 90 seconds after the injection. As the records in addition to the auricular response show a small variation due to the ventricular systole, the actual activity can be gauged by the lengthened P–R intervals. The results are tabulated in Table IV.

TABLE IV. Influence of adenosine upon the absolute refractory period of auricular muscle. Injection into femoral vein.

Dog and controlled heart rate per min.	Absolute refractory period in secs.			Remarks
	Before injection	15–30 secs. after injection	90 secs. after injection	
TD* 220	0·134	0·095	—	50 mg. P–R interval lengthened from 0·08 to 0·10 sec.
TD* 222	0·131	0·090	0·121	50 mg. P–R interval lengthened from 0·08 to 0·11 sec.
UD* 400	0·104	0·084	—	50 mg. 2 : 1 response of ventricle after injection
UD* 230	0·126	0·099	—	50 mg. P–R interval lengthened from 0·07 to 0·08 sec.
UD* 228	0·140	0·080	—	100 mg. P–R interval lengthened from 0·07 to 0·10 sec.
VD* 240	0·124	0·110	0·119	50 mg. P–R interval lengthened from 0·10 to 0·13 sec.
YD* 235	0·128	0·095	—	50 mg. P–R interval lengthened from 0·11 to 0·15 sec.

* Fully atropinized.

From this table it can be seen that the absolute refractory period is consistently shortened, being reduced by some three-hundredths of a second by an injection of 50 mg. and by some six-hundredths by an injection of 100 mg. of adenosine, and that within about 90 seconds of the injection the preinjection value is again restored.

It has been noted that "after-effects of stimulation" such as auricular flutter and fibrillation are readily provoked when the rhythmic and testing shocks are passing through muscle under the influence of these substances. The disordered actions always begin when the testing shock enters the muscle just after the absolute refractory period is ended. At this moment the muscle is apparently in the critical condition which has been fully ascribed and commented upon (10).

The conduction of the excitation wave across the auricle has been tested in the dog's heart. The arrival of the excitation wave at two separate points in the auricular muscle has been recorded electrically and simultaneously by placing two paired non-polarizable electrodes in contact with the muscle and connecting each to a galvanometer string. The auricular rate has been maintained constant throughout each observation by passing rhythmic break shocks through electrodes embedded in the muscle, and the non-polarizable electrodes so placed that the excitation wave passes in a straight line beneath each in turn. In these observations measurements have been made immediately before, 15–30 seconds after and also about 90 seconds after the injection. The results are tabulated below (Table V).

TABLE V. Influence of adenosine upon the conduction of the excitation wave through auricular muscle. Injection into femoral vein.

Dog and heart rate	Conduction interval in seconds: Before	15–30 secs. after injection	90 secs. after injection	Remarks
UD				
250	0·0176	0·0155	0·0176	Electrode distance =16 mm. 50 mg. adenosine injected
380	0·0219	0·0186	0·0191	
VD				
240	0·043	0·041	0·038	Electrode distance =35 mm. 50 mg. adenosine injected
300	0·041	0·038	0·038	
400	0·050	0·037	0·036	
450	0·051	0·049	0·045	
YD				
240	0·034	0·029	0·032	Electrode distance =28 mm. 50 mg. adenosine injected
300	0·035	0·031	—	
420	0·037 (complexes irregular)	0·029 (complexes less irregular)	0·039 (complexes irregular)	—
450	Great irregularity, not measurable	0·034 (complexes less irregular)	—	—
420	Complexes irregular, not measurable	0·033 (complexes less irregular)	0·035 (complexes irregular)	—
300	0·034	0·033	0·034	—

It would appear from these observations that adenosine has no influence upon the rate of conduction of the excitation wave when the auricle is responding to the low rates of rhythmic stimulation. If however the rate of beating is raised, so that each successive excitation wave passes through muscle in which conduction has incompletely recovered, the impaired conduction is somewhat improved. At the same time the irregularity of the electrical complexes which accompanies slow conduction at high rates of beating (9) is slightly but definitely reduced. The influence which the substance exerts upon conduction is explained by the reduction of the absolute refractory period allowing a longer rest period between the successive excitation waves rather than by an effect upon actual fibre conduction. A similar improvement in conduction has been seen after vagal stimulation (9), though in this circumstance, owing to the extremely short absolute refractory period which this stimulation brings about, the improvement is much more definite.

The recovery curve of conduction in muscle under the influence of adenosine has not been specifically investigated, but the recovery curve of the time elapsing between the moment of stimulation and the arrival of the excitation wave at the recording electrode (stimulus to intrinsic interval) has been plotted in several experiments. This interval includes, in addition to the time taken for the excitation wave to travel from the point of stimulation to the recording electrode, the latency of response. It has been shown, however, that under many different conditions (11) this curve follows closely the curve of recovering conduction. Two

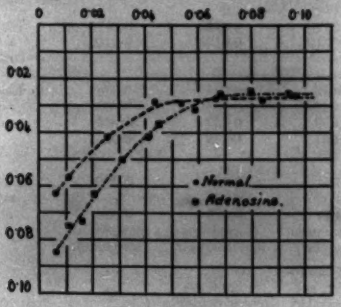

Fig. 2. See text.

typical curves are shown in Fig. 2. The interval in seconds elapsing between the termination of the absolute refractory period and the

extra stimulus which is taken to represent the previous period of rest is represented by the abscissæ, the stimulus to intrinsic interval by the ordinates.

The curves show that during the maximum effect of the substance the premature responses are subject to greater delay, and full recovery is reached about one to two-hundredths of a second later than in the normal animal. If the observed differences in the curves are an expression of conduction change, it is clear why the prolonged conduction intervals seen at the high rates of beating are not more definitely reduced; the longer period of rest available after the termination of the absolute refractory period being counterbalanced by the greater delay in the recovery of conduction.

Influence upon experimentally produced auricular fibrillation and flutter. Auricular fibrillation or flutter has been produced experimentally in several dogs by stimulating the auricle momentarily with a faradic current. The fibrillation or flutter thus produced persists for a short and variable period, the normal rhythm being sooner or later restored. In all experiments a small dose of quinine sulphate has been previously injected, as in these circumstances the experimentally produced fibrillation or flutter persists for a longer period than in the normal animal and allows the influence of the injection to be studied with

TABLE VI. Influence of adenylic acid and adenosine upon experimentally produced auricular flutter and fibrillation.

Dog	Type of tachycardia and ventricular rate	Duration of tachycardia before injection and dose given	Time in secs. after injection to restoration of normal rhythm	Heart rate per min. immediately after restoration of normal rhythm	Heart rate 60 secs. later
0·03 g. quinine sulphate injected.					
LD*	Flutter 228 (reg.)	2′ 20″ 40 mg. (approx.) adenylic acid	17	57	132
0·08 g. quinine sulphate injected.					
MD*	Fibrillation 240 (irreg.)	4′ 30″ 40 mg. adenylic acid	15	42	130
MD*	Fibrillation 220 (irreg.)	2′ 20″ 40 mg. adenylic acid	10	30	108
0·02 g. quinine sulphate injected.					
PD*	Fibrillation 250 (irreg.)	1′ 30″ 40 mg. adenosine	14	48	120
PD*	Fibrillation 240 (irreg.)	2′ 0″ 40 mg. adenosine	15	50	120

* Fully atropinized.

greater certainty. The influence of adenylic acid and adenosine upon these disordered actions has been examined in fully atropinized animals and the results tabulated above (Table VI).

The results show that the arrest of the fibrillation is not fortuitous as it occurs at approximately the same moment after injection in all the animals tested, and also when, as judged by their other activities, the injected substances are exerting their maximum effect. The latter is actually indicated in these observations by the restored sinus rhythm being at first extremely slow, and gradually returning to its normal rate. Fig. 3 is a blood-pressure record (Dog OD) in which the actual termination is recorded. An electrocardiographic record was taken simultaneously

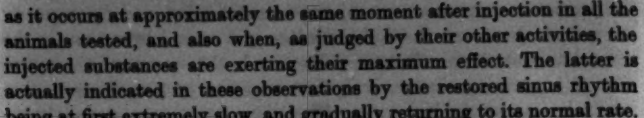

Fig. 3. Dog OD. Termination of auricular fibrillation, experimentally produced, by injection of 40 mg. of adenosine into the femoral vein.

to check the cardiac rhythm throughout. The auricular fibrillation had persisted for about 1 minute 30 seconds before the injection was made. The irregular heart beat and blood-pressure associated with auricular fibrillation are at first recorded; about 10 seconds after the injection the latter begins to fall[1], and 4 to 6 seconds later the irregular heart beat is suddenly replaced by a very slow beat which gradually quickens till a regular rate of 120 per minute is reached. At the same time the blood-pressure rises gradually to a steady and constant level associated with a normal heart beat. The arrest of these disordered actions must be ascribed to a direct action of the substance. It is probable that

[1] The fall in blood-pressure will be discussed later.

the explanation offered for the arrest of auricular fibrillation by vagal stimulation(12) may hold in this case also, as adenosine and vagal stimulation both shorten the refractory period of auricular muscle and improve impaired conduction due to high rates of beating. It has been ascribed in the case of vagal stimulation to the manner in which the refractory period is reduced and to the opposing effects of this shortening upon the length of the responsive gap between the crest of the oncoming wave, fibrillation being considered as a circus movement, and the tail of the receding wave. During the stimulation the oncoming wave travels more quickly, and, finding before it no responsive tissue to enter, dies out. We have already mentioned when dealing with the refractory period of auricular muscle that "after effects of stimulation" are easily produced while adenosine is exerting its effect upon the muscle. In this particular also it simulates vagal stimulation, which makes a common explanation even more probable. On the other hand we have obtained some evidence that adenylic acid and adenosine have not the same action in muscle which has been exposed to the "strain" of fibrillation as in normal muscle. The differences may be one of degree only, but on this account we would not stress unduly the explanation offered above.

The auricular contraction. If the heart is carefully inspected during the injection of these substances the auricle is seen not only to beat much slower but also to contract less forcibly, and the two effects appear to run parallel. To confirm this observation myograph records have been made of the auricular contraction with a Cushny myograph. These records show that in the fully atropinized animal an injection of 30 mg. adenosine, which slows the rate of beating from 170 to 90 per minute, reduces the amplitude of a contraction from 28 mm. to 12 mm., and the greatest reduction in amplitude coincides with the maximal retardation. When the auricle is driven at a constant rate the amplitude is reduced, the greatest reduction recurring about 15 seconds after the injection. Both substances, therefore, definitely influence the contraction process in auricular muscle.

The excitability of auricular muscle. The action of the substances upon excitability has been tested by passing shocks through metal electrodes fixed firmly in the auricular muscle and separated from one another by about 1 mm. of muscle. The threshold value for a series of rhythmic shocks passed through such electrodes at a rate a little above the natural heart rate has been found, and no constant evidence of either a definite fall or a rise in excitability to such shocks has been obtained

after the injections. Judged by this method, which is obviously crude, the substances tested have no definite influence upon the excitability of auricular muscle.

The ventricle. Doses up to 100 mg. of adenosine appear to exert no definite influence upon the properties of ventricular muscle. The absolute refractory period, estimated in the manner originally described (13), is not affected as the following table shows (Table VII).

TABLE VII. Influence of adenosine upon the absolute refractory period of ventricular muscle. Injection into femoral vein.

Dog	Controlled heart rate	Absolute refractory period in secs. Before injection	30 secs. after injection	90 secs. after injection	
UD*	200	0·148	0·152	0·136	50 mg. adenosine
VD*	240	0·156	0·158	0·157	50 ,,
ZD*	200	0·128	0·125	0·129	50 ,,
GAD*	220	0·140	0·139	—	100 ,,

* Fully atropinized.

No actual measurements have been made upon the conduction of the excitation wave through the ventricular muscle after injection. The electrical complexes, both from lead II and from direct leads, show no deformation either when the heart is beating naturally or when it is being rhythmically driven at rates of 200 per minute by shocks applied to the ventricular muscle. In addition the stimulus-intrinsic intervals are unchanged. These results are incompatible with any definite change in conduction. No change can be detected by the eye in the contraction of the ventricle responding to rhythmic shocks. The amplitude of a right ventricular myogram, obtained by the Cushny myograph, from a heart responding to rhythmic shock liberated from a constant point in the ventricular muscle remains unchanged (14), while in the naturally beating heart the amplitude is definitely increased when the heart beats more slowly. These observations show that adenosine has no influence upon the strength of contraction of ventricular muscle. A change in direction in the *T*-wave is very frequently, though not invariably, seen after the injection of either adenosine or adenylic acid. This change does not coincide with the period of maximum activity, but appears about 30 seconds after the injection, and is not fully developed till 30 seconds later. At this stage the pre-injection heart rate has been restored, and it is not therefore a rate effect. The change persists for about 2–3 minutes and is independent of the original direction of the wave. We can offer no explanation of this change. On several

occasions ventricular fibrillation has been produced by faradizing the ventricles momentarily. Adenosine, in doses up to 250 mg., has been injected into the left ventricular cavity immediately the disordered action has commenced, and the heart has been massaged. Such a procedure has had no effect on the fibrillation.

The coronary flow. The influence of adenosine upon the outflow from the coronary sinus[1] in the dog's heart has been measured in a heart-lung preparation, a very definite increase in flow being recorded. The adenosine has been injected into the tube leading to the superior vena cava and the heart driven rhythmically to avoid any effect of changing rate. An injection of 10 mg. of adenosine increases the flow from 41 c.c. per minute to a maximum of 200 c.c. per minute, while 5 minutes later the outflow still measures 83 c.c. per minute. After atropinization the outflow is increased by the same dose from 48 c.c. per minute to a maximum of 300 c.c. per minute, falling to 160 c.c. per minute at the end of 10 minutes. The aortic blood-pressure during these observations has been maintained constant at 82 mm. Hg. In such a preparation therefore the coronary vessels are dilated by small doses of adenosine, the degree of dilatation being that produced by sodium nitrite (15). The dilatation persists for about 15 minutes while the sinus bradycardia which also occurs lasts only for 2 minutes. In the whole animal the general blood-pressure is lowered by the substances so that the great increase in coronary outflow seen in the above experiment would not be expected, but it can be safely assumed that the coronaries are dilated[2] and that the dilatation persists for a longer period than the sinus bradycardia.

Influence upon the arterial pressure.

The influence upon the arterial blood-pressure has been repeatedly tested in both cats and dogs. A fall in pressure is constantly recorded (Fig. 4) which is independent of vagal section or atropinization. In a dog of 6 kg. an injection of 40 mg. usually lowers the pressure by about 50 mm. Hg. The fall is not due solely to the cardiac slowing, for an injection of 40 mg. into the aorta which does not retard the heart rate still lowers the blood-pressure some 25 mm. Hg. The fall in pressure is

[1] We are indebted to Dr Anrep for performing this experiment for us.

[2] Before these observations were made, we were of opinion that, at the end of an experiment involving many injections, the coronary vessels stood out unusually clear, and we suspected coronary dilatation. We also considered the hearts in an unusually good condition throughout. The latter is perhaps to be ascribed to the coronary dilatation.

therefore in part due to a dilatation of arterial vessels and in part to the slowing of the heart beat. Observations upon the pulmonary pressure

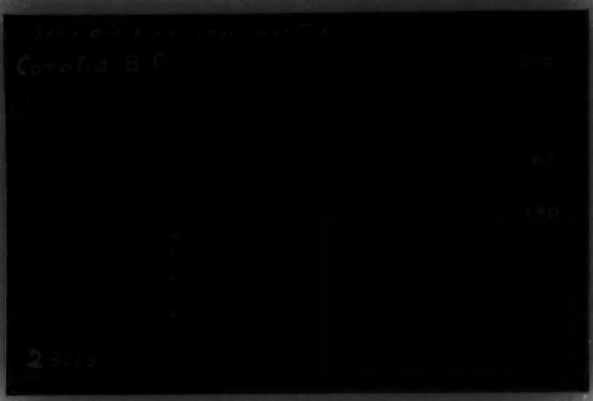

Fig. 4. Dog PD. Influence of injection of 40 mg. adenosine into femoral vein upon blood-pressure and heart rate.

in the heart-lung experiment show that adenosine produces a rise in pulmonary pressure. This rise is explained by the opening up of the coronary vessels which short-circuits the blood from the systemic to the pulmonary circulation, and not by a constriction of the pulmonary vessels.

The contraction of skeletal muscle.

Embden and his co-workers (4, 5, 6) consider that the deamination of adenylic acid is one of the earliest events in muscular activity. We have therefore investigated the action of adenosine upon the contraction of the tibialis anticus in the cat to determine its influence upon the contraction process itself. The left tibialis anticus has been tetanized intermittently by the faradic stimulation of its nerve; the period of tetanus and rest being approximately 1 second each. The contraction of the muscle has been recorded by exposing the tendon and connecting it to a spring lever writing on a smoked drum. The injection has been made into a cannula placed in the right iliac artery close to its origin from the common iliac artery so that the adenosine passes directly into the circulation of the left leg. When a muscle is tetanized in the manner

described, the contraction rapidly decreases, till a steady amplitude is reached, which persists for a considerable time. The injection of 20 mg. of adenosine during the steady state has no influence upon the amplitude of contraction. If the dose is increased to 50 mg. a transient diminution of the contraction is seen. We are not inclined to ascribe this to a specific action of the adenosine upon muscle contraction, for with such an injection the heart is slowed and there is a fall in general arterial pressure, which in itself, as Florey and Szent-Györgyi(16) have shown, leads to a decreased amplitude of contraction. If the tetanus period is lengthened and the rest period shortened, the amplitude of contraction steadily decreases, the muscle becoming more and more fatigued. Intra-arterial injections of adenosine up to 15 mg. during this period have no influence upon the amplitude of contraction; greater doses are accompanied by general arterial pressure changes which complicate the results. The method of injection employed ensures that the muscle tested receives the dose of adenosine before it is reduced by passing though organs such as the liver, etc., and it would appear from these observations that it has no effect upon the contraction process itself.

Intestinal movements. The influence of adenosine upon the movements of the small intestine has been examined in cats by the balloon method. The injection of 25 mg. of adenosine into the femoral vein completely and abruptly inhibits the movements; the inhibition lasting about 2 minutes. The movements return equally abruptly and at first are slightly larger than before the injection. Upon re-injection the inhibition can be repeated. Full atropinization does not influence the result. Experiments upon the rabbit's intestine suspended in Tyrode solution show that the amplitude of the contractions is considerably reduced when the concentration of adenosine in the solution reaches 0·002 p.c., while the rhythmicity is but little affected.

The secretion of urine.

Although adenylic acid is apparently present in kidney tissue in similar concentration to that in other tissues, the influence of adenosine upon the secretion of urine in the heart-lung-kidney preparation[1] is only definite when large doses are employed. Injections up to 25 mg. have no effect, but if 100 mg. doses are used the secretion is momentarily greatly reduced though the general blood-pressure be maintained constant. Coincident with this decrease the blood flow through the kidneys is reduced. The composition of the urine during the period of

[1] Our thanks are due to Prof. Verney for performing this experiment for us.

decreased secretion is qualitatively the same as would be produced by a fall in perfusion pressure. At the moment we have no evidence of an action upon the kidney cells as the results obtained can be explained by an action upon the blood vessels leading to a fall in the glomerular capillary pressure.

The influence of temperature.

The temperature of the animal has a considerable influence upon the activity of adenosine and adenylic acid. It has been found consistently in the guinea-pig that the heart block lasts three times longer at a temperature of 34° C. than it does at 37° C., for the same dose. The intensity of the reaction as well as the duration is increased; the results at low temperatures being duplicated at high temperatures only by injecting much larger amounts of the substance. It is essential therefore that the animal's temperature should be maintained constant while estimating the amount of substance present in any fraction, for considerably more substance will be estimated at low than at high temperatures. This change in activity which amounts to 200–300 p.c. for a drop of 5° C. is to be ascribed to the low temperature producing in itself an action upon the heart similar to that of adenosine. When the temperature of the guinea-pig is lowered to 32° C. the heart rate is reduced by about 100 beats a minute but the P–R interval is already lengthened. The junctional tissue is apparently more affected by cold than the sinus node, so that conduction from auricle to ventricle is already impaired and the increased activity registered at low temperatures due to the summing of these two actions.

The influence of barium, calcium, potassium and phosphotungstic acid.

We had repeatedly noted during the isolation that the activity of fractions which contained, in addition to the responsible constituent, a trace of barium was definitely weaker than the same fractions completely free from barium. We have consequently tested in several animals the influence of barium upon the heart block produced by adenosine and adenylic acid.

The experiments consistently show that after an intravenous injection of 1 mg. of barium acetate which enhances the heart rate a little or leaves it unchanged and has no influence upon the electrical record, 0·1 mg. of adenosine no longer produces heart block. At this stage 0·2 mg. of the substance produces a heart block of very short duration, which can be abolished by a further injection of barium. Various salts

of barium have been tested and all have this antagonizing action, clearly indicating that the barium ion is responsible. The reverse experiment has also been carried out, a persistent incomplete heart block has been produced in a guinea-pig by the injection of 50 mg. of adenosine subcutaneously, and barium chloride has been subsequently injected intravenously. In such an experiment, 2–3 mg. of barium chloride temporarily abolish the block; it reappears about 2 minutes later, and can be repeatedly abolished by further injections.

Calcium chloride injected intravenously in doses of 4 mg. and potassium chloride similarly injected in doses of 4 mg. have no effect on the duration of the block produced by 0·2 mg. of adenosine injected immediately after. Phosphotungstic acid, on the other hand, intensifies the reaction; an intravenous injection of 2 mg. doubling the duration. The P–R interval, however, is lengthened by the acid itself, showing that it has a similar action to adenosine, and the increased duration is due to the summing of the two effects. With regard to the action of barium, it apparently renders the junctional tissues less susceptible to the action of adenosine and adenylic acid. We have no evidence whether this is due to a specific action upon the chemical deamination, which underlies the activity of these substances, or to an action upon a separate mechanism in the contrary sense to these substances.

Action in the unanæsthetized animal.

Adenylic acid and adenosine have been injected in doses up to 100 mg. intravenously into the normal rabbit, and both produce a very transient bradycardia, during which the animal appears apprehensive and listless. Adenylic acid is absorbed from subcutaneous injections with difficulty, doses up to 20 mg. in the guinea-pig having no influence upon the heart, while a few dropped beats only are seen 5 minutes after the injection of 60 mg. Adenosine, on the other hand, is readily absorbed; for within 50 seconds of a subcutaneous injection of 5 mg. dropped beats are seen. An injection of 50 mg. is followed by a heart block which may be a 4 : 1 or 3 : 1 block or the auricle and ventricles completely dissociated. The auricular rate is retarded to about 200 per minute and the ventricular rate to 60–90 per minute. The effect is maximum in about 5 minutes, and gradually passes off, the normal rhythm being restored about 1 hour later. If the injection is repeated similar effects are observed. The animal immediately after the injection appears normal, but within a minute or two develops a panting respiration, becomes listless, and tends to lie quite still and on occasions to sleep. The animal is normal

again within an hour. Whether the low blood-pressure, the heart block and the loss of intestinal movement are sufficient to bring about the observed condition or whether an action on the nervous system must be considered we are unable to state. A subcutaneous injection of 50 mg. of adenosine twice daily for a week in the guinea-pig[1] and one of 12 mg. twice daily for a week in the mouse has no effect. The lethal dose of adenosine for the latter is 3 g. per kg. subcutaneously injected. Both substances seem to have no toxic action and are not cumulative. Adenosine has been injected subcutaneously into the healthy man in doses up to 1 g. with no ill effects (17).

Summary.

1. Simple extracts from heart muscle, brain, kidney and spleen have a definite and transient effect upon the mammalian heart.

2. The substance responsible for this action has been isolated and appears from its chemical properties to be adenylic acid. Its activity apparently depends upon the ease with which the substance is deaminated in the body.

3. Adenosine, prepared from yeast nucleic acid, has an action identical to adenylic acid.

4. The physiological activity of both substances has been tested by intravenous injection into the whole animal.

5. Among other effects upon the heart, they slow the rate of beating, impair conduction from auricle to ventricle, and arrest experimentally produced auricular fibrillation; they shorten the absolute refractory period of and improve slowed conduction in the auricle due to high rates of beating.

6. They lower general arterial pressure. This is due in part to the cardiac slowing and in part to a general arterial dilatation. They dilate the coronary vessels and inhibit intestinal movements.

[1] Examination of the blood shows that there is no change in either the red cell and the white cell counts or in the hæmoglobin content, nor any evidence of pathological change at the end of seven days.

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THE ANTI-DIURETIC EFFECT OF THE SEPARATED PRINCIPLES OF THE PITUITARY BODY.

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The separation of the pressor and the oxytocic principles of the posterior lobe of the pituitary body by Kamm, Aldrich, Grote, Rowe and Bugbee(1) has been confirmed by several observers.

The separated principles, pitocin and pitressin, have also been investigated with regard to their possession of the anti-diuretic properties associated with whole extracts of the posterior lobe of the pituitary body. This anti-diuretic action is best shown in the inhibition of the diuresis consequent upon the ingestion of large quantities of water, in the inhibition of diuresis in diabetes insipidus and in controlling the output of urine from a kidney free from the influence of the pituitary body as in the heart-lung-kidney preparation of Starling and Verney(2). Burn(3) has investigated the inhibitory action of pitocin and pitressin on water diuresis in man and reports that while small amounts (0·02 c.c.) of pitressin are able to stay the onset of the diuresis following the ingestion of a litre of water, there is no such action associated with pitocin. Gargle, Gilligan and Blumgart(4) report that pitocin is not able to inhibit the diuresis of diabetes insipidus to the same extent as pitressin.

Certain observations, however, suggested that some degree of anti-diuretic action might be associated with pitocin, and experiments have been performed to explore this possibility. Two methods of investigation have been employed. In the first, the effect of the principles in regulating the output and concentration of urine in a heart-lung-kidney preparation has been determined and, using this technique, there was the additional possibility of observing and controlling any changes in the vascular conditions of the kidney. In a second series of experiments, an examination has been made of the relationship which exists between different amounts of the principles and the extent to which they are able to inhibit water diuresis in man.

The action of the separated pituitary principles on urine formation in the heart-lung-kidney preparation.

The apparatus employed in these experiments was arranged substantially as described by Starling and Verney, but minor modifications were made, particularly in the control of the venous inflow to the heart-lung preparation.

To avoid undue strain of the heart, the perfused kidney was placed about 30 cm. below the level of the heart-lung preparation, a high renal blood-pressure being thus available without any corresponding increase in cardiac work.

Venous blood which issued from the kidney was collected in a funnel which led, together with blood coming from the heart-lung circuit, into a large venous reservoir. Blood was pumped from here at a rate exceeding the cardiac output to a subsidiary reservoir situated above the heart. A constant level of blood was maintained in this second reservoir by allowing blood pumped in excess of the requirements of the heart to return by an overflow tube to the main reservoir. The actual inflow to the heart was controlled by a screw clip on the tube leading to the cannula in the superior vena cava.

As far as possible all the apparatus was accommodated in a large water bath kept at constant temperature. The subsidiary venous reservoir had a water jacket through which water was circulated from the main bath by means of an "Autopulse." A thermometer in the renal cannula recorded the temperature at which blood entered the kidney and the rate of blood flow was measured by a Stolnikov stromuhr fitted in the kidney circuit immediately before the cannula. The pressure of perfusing blood was measured on a mercury manometer connected to a side tube of the renal cannula. By adjustment of the variable resistance in the heart-lung circuit, the renal pressure was kept constant throughout an experiment and any changes in the vascular condition of the kidney were estimated as variations in blood flow.

Preliminary experiments with a heart-lung preparation showed that 0·1 c.c. of pitressin or pitocin, added to 700 c.c. of circulating blood, had no effect on heart output or aortic pressure, although pitressin caused a very small and transient diminution in cardiac volume.

Eight experiments have been performed with the kidney in circuit, four with each extract, and in all cases an anti-diuretic effect has been obtained. The results of two experiments are depicted in Figs. 1 and 2. As with the addition of unseparated pituitary extracts to the circulating

blood of the heart-lung-kidney preparation, so with pitocin and pitressin the output of urine is reduced and there is an increase in the concentration of urinary chlorides. In the experiment represented by Fig. 1,

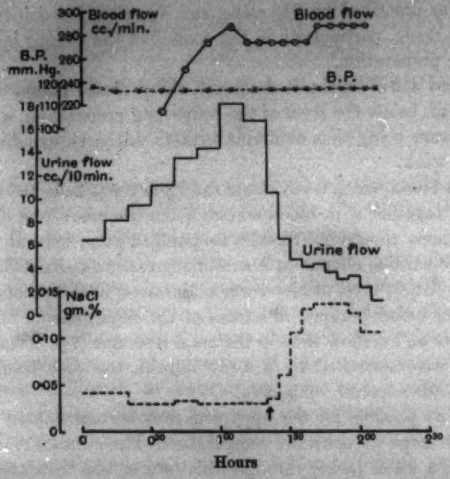

Fig. 1. Effect of pitocin upon output of urine and concentration of urinary chloride in heart-lung-kidney preparation. The arrow indicates the point of addition of 0·1 c.c. of pitocin to 1300 c.c. of circulating blood. Temperature of renal blood = 36° C. Wt. of kidney = 36 g.

following the introduction of 0·1 c.c. of pitocin into approximately 1300 c.c. of circulating blood, there was a gradual reduction in urine flow from 16·5 c.c. per 10 minutes to 1·0 c.c. per 10 minutes with a concomitant increase in the concentration of urinary chloride (estimated as NaCl by Volhard's method) from 0·03 g. p.c. to 0·13 g. p.c. There was no diminution in blood flow, but on the contrary a slight increase in the rate, the blood-pressure being maintained constant. The addition of pitressin to the preparation, however, always resulted in a diminution of renal blood flow immediately following its introduction, indicating vasoconstriction, since the artificial resistance was manipulated to keep the pressure constant. The urinary changes induced by pitressin were also more pronounced than with pitocin. Thus from Fig. 2 it will be seen that although only 0·05 c.c. of pitressin was added to the same volume of

blood as in the experiment of Fig. 1, the urine flow was reduced much more rapidly and the chloride was increased to a great extent. Similar results were obtained in other experiments.

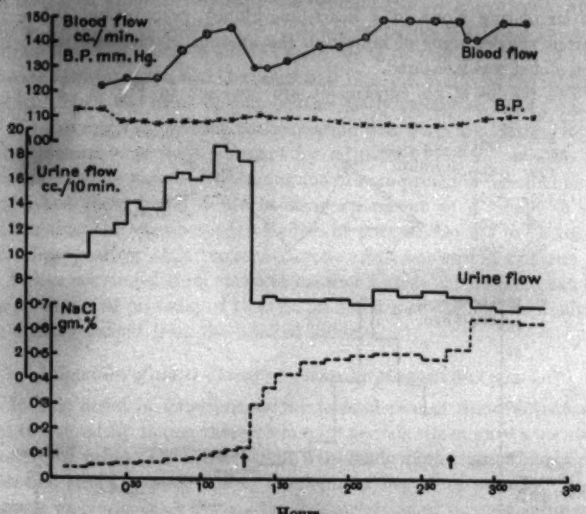

Fig. 2. Effect of pitressin upon output of urine, concentration of urinary chloride and renal blood flow in the heart-lung-kidney preparation. The arrows indicate the points of addition of 0·05 c.c. of pitressin to 1300 and 1100 c.c. respectively of circulating blood. Blood-pressure controlled by artificial resistance. Temperature of renal blood = 35° C. Wt. of kidney = 26 g.

It was not possible to assess exactly the relative potency of the extracts since the experiments are not strictly comparable, but it appeared from the results obtained that the anti-diuretic action of pitressin was at least three times as powerful as that of pitocin. However, following experiments which will be discussed later, on the inhibition of water diuresis in man, it was possible to make a much more accurate comparison of the relative potency of the extracts.

Determination of the minimum amount of pitressin required to arrest diuresis in the isolated kidney.

A dilute solution of pitressin was prepared and allowed to run into the circulating blood of a heart-lung-kidney preparation. When the diuresis showed signs of inhibition the amount of pitressin which had been added was measured.

The results of an experiment are depicted in Fig. 3. 1·0 c.c. of

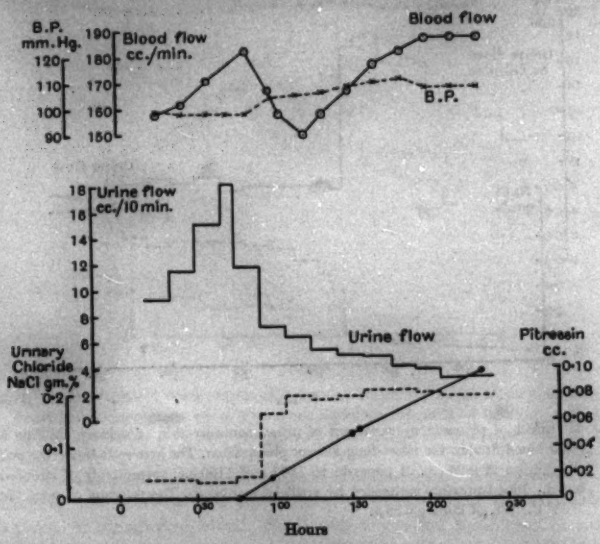

Fig. 3. Effect of slowly adding a dilute solution of pitressin to 1600 c.c. of circulating blood. Ordinate on the right represents amount of pitressin added. Wt. of kidney = 51 g.

pitressin was diluted and made up to 250 c.c. with saline so that each c.c. of the solution contained 0·004 c.c. of the extract. By means of a burette the solution was slowly added to the venous reservoir feeding the heart-lung preparation, and thorough mixing with all the blood was ensured because of the overflow and circulation from the main venous reservoir. The burette was only permitted to empty over a range of 10 c.c. before refilling so that the rate of inflow might be kept constant. In the period of the experiment immediately before the infusion of

pitressin was commenced the rate of urine formation was 18·2 c.c. in 10 minutes and the chloride concentration of the urine equivalent to 0·035 g. p.c. NaCl. The rate of urine formation and rate of blood flow had increased progressively from the beginning of the experiment. After adding 0·018 c.c. of pitressin to 1600 c.c. of circulating blood the rate of urine flow had fallen to about two-thirds of its original value and the chloride concentration was increased to 0·16 g. p.c. NaCl. As the infusion was continued the urine output became fairly steady at 3·0–4·0 c.c. in 10 minutes and the chloride concentration was 0·2 g. p.c. NaCl. Renal blood-pressure was kept constant by adjustment of the variable resistance, but the vaso-constrictor action of vaso-pressin is reflected in the diminished renal blood flow. In this experiment as in others it was noticed that the vaso-constrictor effect of pitressin tended to be transitory, disappearing while the anti-diuretic effect was still in evidence.

It was concluded from this and another similar experiment that less than 0·01 c.c. of pitressin in 1600 c.c. of blood was sufficient to diminish the urinary output from the isolated kidney.

Inhibition of water diuresis in man by pitressin and pitocin.

In this series of experiments the normal rate of urine output was first measured by taking three 15-minute periods which gave a constant amount of urine. The observations were made at the same time of day with the subjects maintaining a minimum degree of activity. Water diuresis was induced, following the determination of the normal urinary output, by drinking one litre of water in 15 minutes. This amount was usually sufficient to increase the urine output within 20 minutes and diuresis was fully established 45 minutes after the ingestion of the water (Fig. 4 *a*). Immediately following the ingestion period, pitocin or pitressin was injected subcutaneously in the required quantity. For each experiment the pitocin or pitressin was diluted with saline so that the volume required for injection was contained within 0·5 c.c. of solution.

The anti-diuretic effect of different amounts of the extracts was assessed by the extent to which the onset of diuresis was postponed and by the reduction in maximum rate of urine output compared with the rate in a normal diuresis. Results obtained in a number of experiments indicate that pitressin and pitocin possess a similar anti-diuretic action on water diuresis in man to that which they show on the perfused isolated kidney. The actual experiments described were performed on one of us (A. H.), but similar results have been obtained on others.

The amounts of pitressin and pitocin used have been given as absolute volumes since the units in which they are assessed by the makers have

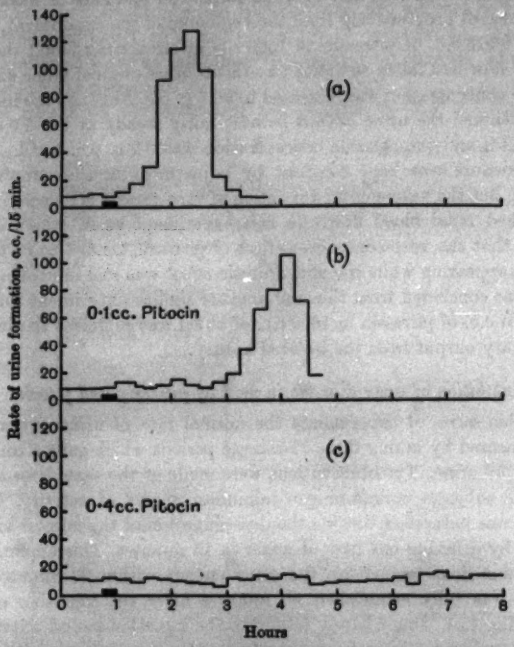

Fig. 4. (a) Normal diuresis consequent on the ingestion of one litre o fwater, (b) and (c) show delay in onset of diuresis following injection of different amounts of pitocin. Water taken during the period represented by shaded area.

reference to their pressor and oxytocic activities respectively and are not concerned with any anti-diuretic properties which may be possessed.

In preliminary experiments relatively large amounts (0·5 c.c.) of either extract were found to cause almost complete inhibition of diuresis as depicted for pitocin in Fig. 4 *c*. Burn has suggested that pitocin does not possess any anti-diuretic activity, but it has not been possible to confirm this absolutely. If a sufficiently large amount of pitocin be given there can be no doubt regarding its inhibitory properties, but as a

measure of potency we took the volume of the extract which was able to delay the onset of diuresis for 2 hours from the end of the water ingestion period. Fig. 4 *b* shows that 0·1 c.c. of pitocin possessed this power. A similar volume of pitressin, however, delayed diuresis for over 7 hours (Fig. 5 *d*) and the action of smaller amounts is depicted in the

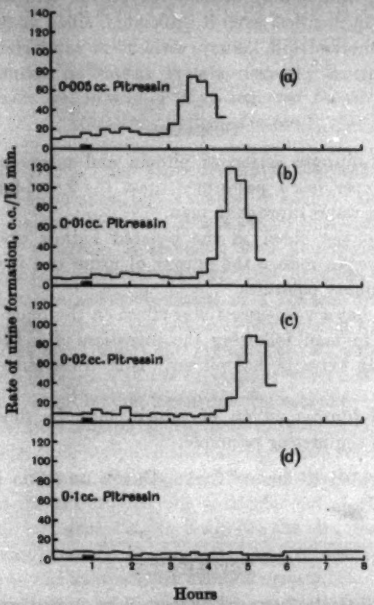

Fig. 5. Effect of pitressin in delaying the onset of diuresis following ingestion of water. (*a*), (*b*), (*c*) and (*d*) show the effect of different amounts of pitressin.

various curves, Fig. 5 *a*, *b* and *c*, from which it will be seen that 0·005 c.c. of pitressin was equivalent to 0·1 c.c. of pitocin in its ability to delay the onset of diuresis.

Thus both extracts possess anti-diuretic properties, but pitressin is effective in one-twentieth of the amount required of pitocin. Since this difference in the amounts of the principles required to produce the same effect is so great, it is necessary to consider whether the action of pitocin may not be due to the presence of small amounts of pitressin as impurity.

Gaddum(5) in an analysis of some of the properties of these substances has shown that 1·0 c.c. of pitocin may possess slightly less than one international unit of pressor activity, while 1·0 c.c. of pitressin contains 20. This is the ratio in which the anti-diuretic property is divided, and therefore the slight anti-diuretic action of pitocin may be due to imperfect separation. But it may be that the pressor and the anti-diuretic properties are not identical even if associated, since pitocin inhibits the urine flow in the isolated kidney without any interference with the blood flow, whereas pitressin always causes an accompanying vaso-constriction.

SUMMARY.

1. The anti-diuretic action of pitocin and pitressin has been investigated in experiments performed upon the heart-lung-kidney preparation and on water diuresis in man.

2. Added to the blood in the isolated kidney preparation both pitocin and pitressin reduce the output of urine and increase the concentration of urinary chlorides.

3. Pitressin has a vaso-constrictor effect on the renal vessels.

4. Diuresis in man following the ingestion of a litre of water is inhibited by both extracts, but pitressin is 20 times more powerful than pitocin.

5. The anti-diuretic action of pitocin may be due to imperfect separation from the pressor principle.

We are indebted to Messrs Parke, Davies and Co., for samples of pitocin and pitressin.

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THE ACTION OF EPHEDRINE ON THE BLOOD-SUGAR [WITH A NOTE ON ITS ACTION ON THE DENERVATED PUPIL OF A CAT].

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It was found by Barger and Dale(1) that the sympathomimetic action is not peculiar to adrenaline, but is shared by a large number of primary and secondary amines. However, it was shown that the approximation to adrenaline in structure is attended with increasing intensity and specificity of sympathomimetic activity. The optimum carbon-skeleton for sympathomimetic activity consists of a benzene ring with a side-chain of two carbon atoms, the terminal one bearing the amino-group. Another optimum condition is the presence of two phenolic hydroxyls in the 3 : 4 position relative to the side-chain: when these are present an alcoholic hydroxyl still further intensifies the activity.

Previous investigations of their various physiological properties have dealt mainly with their action on the blood-pressure and isolated organs, although their effect on the sugar content of the blood has been described. Morita(2) has studied this particular action of the sympathomimetic amines, and according to Nagel(3) the elevation of the blood-sugar content can generally be considered as a measure of the influence of a substance on the sympathetic nervous system.

Since the production of insulin with its lowering of the level of the blood-sugar, considerable attention has been paid to the so-called anti-insulin effect of different substances.

Banting, Best, Macleod, Collip(4) and others have investigated the antagonistic effect of adrenaline and insulin, especially in regard to their effects upon the blood-sugar. Before the investigations of these authors the most convincing work on the antagonism of extracts made from the adrenal glands and that from pancreas is that of Achard, Ribot and Binet(5).

Ross and Davis(6) performed experiments on dogs, from which they conclude that adrenaline and the pancreatic hormone contained in fresh

pancreas preparations react together in an antagonistic balance, the surplus of the larger quantity exerting its influence. Lyman, Nicholis and McCann(7) by injecting both insulin and adrenaline showed an antagonistic effect in their action on the level of blood-sugar.

The purpose of the present paper is to study the action on the blood-sugar of rabbits of ephedrine, ephetonine, tyramine and isoamylamine and to find out whether there exists an anti-insulin action of ephedrine in rabbits and dogs.

The study of the pharmacological action of ephedrine, the chemical structure of which is very closely related to adrenaline by Miura(8), Chen and Schmidt(9), Chen and Meek(10), Leyko(11), Poos(12), Kreitmair(13) and others has proved its strongly specialized sympathomimetic actions. The effects of ephedrine on the circulation, intestines, bronchi, pupil, isolated sphincter muscle of the iris, agree in every respect with those of adrenaline. Evidence was brought forward by Curtis(14) that the pressor action of ephedrine like that of adrenaline is reversed by ergotamine.

Some investigators have tried to determine whether the similarity of action of ephedrine and adrenaline is shown on glycogen metabolism.

Nagel, studying the influence of this substance on the blood-sugar level of rabbits, found that intravenously a dose of ephedrine of about 10 mg. per kg. had an insignificant effect on blood-sugar, while a dose of about 30 mg. per kg. gave a definite increase of blood-sugar concentration, which however was less marked than that produced by an intravenous injection of 0·1 c.c. of 1 to 1000 adrenaline. The results obtained by Wilson(15) confirm the conclusion of Nagel that small doses of ephedrine are without any significant effect on the blood-sugar, while amounts as large as 25 mg. per kg. of rabbit definitely raise the sugar content of the blood. He also found that less ephedrine is required to elevate the blood-sugar level in dogs than in rabbits.

Ephetonine, a substance which agrees in chemical composition with ephedrine but is optically inactive, was studied by Kreitmair(16), who found that the racemic compound possesses qualitatively and quantitatively the same pharmacological action as the natural ephedrine, as judged by their effects upon the blood vessels in the normal or in the decapitated cat, on the heart, also on the uterus, on the intestines and on the size of the pupils. On the other hand the data given by Chen(17) indicate that the average ratio of the intensity of pressor action of synthetic ephedrine to that of natural ephedrine with optimal doses, compared indirectly in pithed cats with adrenaline, is 1 to 1·33. The average

ratio of the mydriatic action of synthetic ephedrine to that of natural ephedrine in Caucasians measured by the increase in the transverse diameter of the pupil is 1 to 1·29. The hyperglycæmia following the administration of synthetic ephedrine is much less than that observed after the subcutaneous injection of adrenaline.

The action of tyramine and isoamylamine has been investigated very thoroughly by Dale and Dixon(18). Both isoamylamine and tyramine have an action which is very similar to that of adrenaline, reproducing both the motor and inhibitory effects of nerves of the true sympathetic system. Their action differs from that of adrenaline in being weaker, tyramine about one-twentieth and isoamylamine by comparison with tyramine roughly about one-eightieth that of adrenaline, and being less strictly though still mainly peripheral.

Method.

The normal well fed animals used in these experiments were starved for 24 hours. Samples of blood were taken from the ear vein in rabbits and from the knee vein in dogs. The solutions of the substances studied were injected into the ear veins except some instances where they were applied subcutaneously. Sugar estimations were made by the method of Hagedorn and Jensen(19).

Results.

The results are shown graphically in the following ten figures, in which ordinates give blood-sugar in gr. per 100 c.c., percentage increase in blood being indicated in brackets; abscissæ, time in hours after the injection, which is marked by an arrow.

Fig. 1 shows that 1–8 mg. of ephedrine per kg. injected intravenously into the rabbit have no effect on its blood-sugar level. Even 30 mg. per kg. of ephedrine increase the blood-sugar content of the same rabbit only by 20·5 p.c.

It is seen from Fig. 2 that the dose of 30 mg. of ephedrine per kg. is as effective as a bigger one such as 50 mg. per kg. which showed almost the same increase of blood-sugar in the rabbit.

Generally speaking the effect of ephedrine on blood-sugar in rabbits is very slight and not exceeding about 26–28 p.c. In one experiment only ephedrine increased the blood-sugar in 2½ hours till above 0·385 per 100 c.c., and this was when the dose reached 80 mg. per kg., a dose which proved fatal. The effect of the synthetic ephedrine is shown in

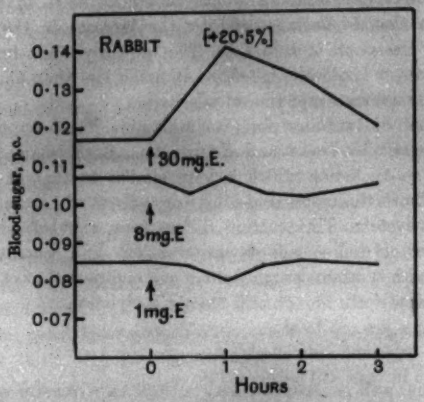

Fig. 1. The effect of different doses of ephedrine hydrochloride per kg. injected intravenously on blood-sugar level of a rabbit.

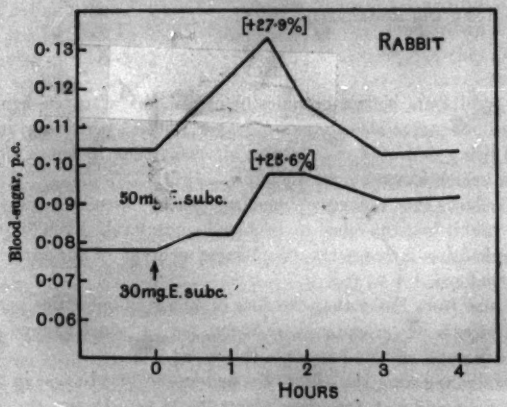

Fig. 2. The action of 30 mg. and 50 mg. of ephedrine per kg. injected subcutaneously on blood-sugar of a rabbit.

Fig. 3. It is seen that the racemic compound has almost the same hyperglycæmic action in the rabbits, the percentage increase of blood-sugar after the same doses as that of natural ephedrine being practically

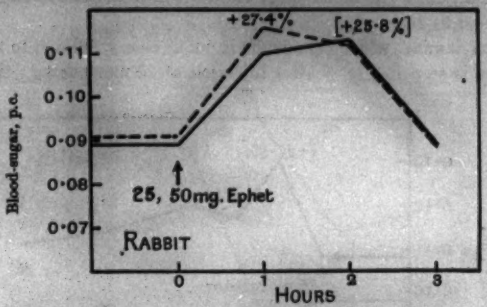

Fig. 3. The influence of 25 mg. (solid line) and of 50 mg. (dotted line) of ephetonine per kg. on blood-sugar of a rabbit.

identical. This confirms the results obtained by Kreitmair on the pressor action and other organs of these substances.

Fig. 4 shows the behaviour of blood-sugar of three rabbits after

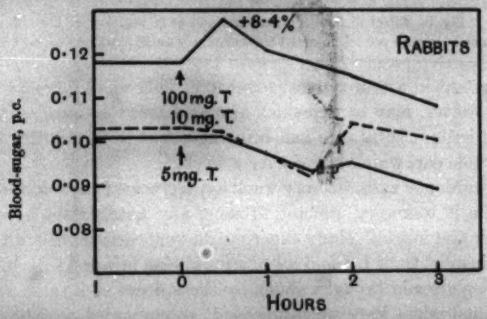

Fig. 4. Influence of the intravenous injection of 5 mg. (lower line), 10 mg. (dotted line) and 100 mg. (upper line) of tyramine per kg. on blood-sugar of rabbits.

intravenous injection of different doses of tyramine. The blood-sugar level even after as much as 100 mg. per kg. does not show greater differences (+ 8·4 p.c.) than may be observed in a normal animal.

The very weak effect of tyramine on glycogenolysis has been described by many authors. Lately Kageyama[20] has stated that only the continuous slow intravenous injection of 0·05 p.c. tyramine into rabbits may raise the blood-sugar from 0·1 to 0·17 per 100 c.c., and the urine sugar to 2 p.c.

Isoamylamine, which chemically is not so closely related to tyramine, raises, as is seen in Fig. 5 after injection of 25 mg. per kg., the blood-

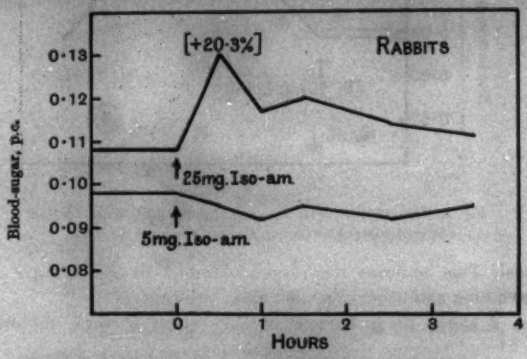

Fig. 5. Effect of 5 mg. (lower line) and of 25 mg. (upper line) of isoamylamine per kg. injected intravenously on blood-sugar of rabbits.

sugar content in a rabbit by about 20 p.c. This small increase of blood-sugar, however, may be explained also as a reflex action, isoamylamine being an irritant substance and bringing about in 20 minutes a swelling of the whole ear, which lasted over a week.

In accordance with the very weak hyperglycæmic action of ephedrine in rabbits it was very difficult to show any antagonism between this substance and insulin. Many experiments were carried out with different doses of insulin, from 1, $\frac{1}{2}$ and a $\frac{1}{4}$ of a unit being injected subcutaneously. The hypoglycæmia brought about by these doses of insulin was not at all modified when they were preceded, accompanied or followed by a large dose of ephedrine, as is demonstrated in Fig. 6. A weak but a distinct anti-insulin action of this substance was observed only when a very small dose of insulin, namely $\frac{1}{8}$ of a unit per kg., was applied and followed by the usual dose of ephedrine. This antagonism, demonstrated in Fig. 7, is shown by the arrest of the fall in blood-sugar after ephedrine

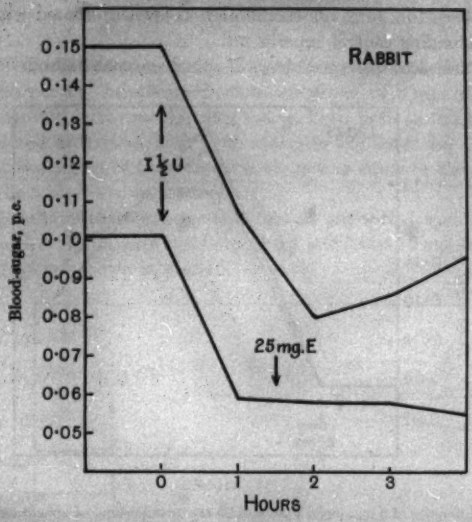

Fig. 6. Effect of $\frac{1}{4}$ unit of insulin (Burroughs, Wellcome and Co.) per kg. injected subcutaneously on blood-sugar of two rabbits. At lowest arrow intravenous injection of 25 mg. of ephedrine per kg.

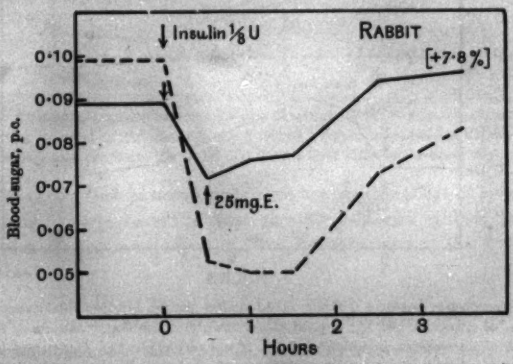

Fig. 7. The antagonistic action of $\frac{1}{8}$ of a unit of insulin per kg. and 25 mg. of ephedrine per kg. (solid line) on blood-sugar of a rabbit. Dotted line indicates the effect of $\frac{1}{8}$ unit of insulin on blood-sugar level without ephedrine.

and its return to a little above the normal level compared with that of the corresponding control experiment.

Fig. 8 indicates that small doses of ephedrine such as from 5 to 20 mg.

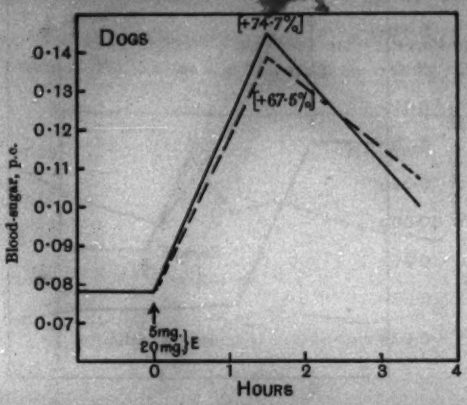

Fig. 8. Influence of 5 mg. (solid line) and 20 mg. (dotted line) of ephedrine per kg. injected intravenously, on the blood-sugar of two dogs.

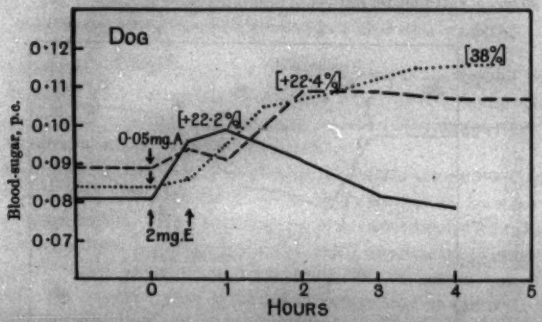

Fig. 9. Synergismus between 0·05 mg. of adrenaline per kg. injected subcutaneously and 2 mg. of ephedrine per kg. applied intravenously in the dog. *Continuous line:* The effect of intravenous injection of 2 mg. of ephedrine per kg. *Interrupted line:* The effect of subcutaneous injection of 0·05 mg. of adrenaline per kg. on blood-sugar content of the same dog. *Dotted line:* The effect of the same doses of both substances on the blood-sugar of the same dog.

per kg. elevate the blood-sugar level in dogs from 65 to 74 p.c. in 1½ hours.

Fig. 9 shows first the separate hyperglycæmic effects of 0·05 mg. of adrenaline per kg. injected subcutaneously and of 2 mg. of ephedrine per kg. injected intravenously into a dog. Then both in the same doses were injected at half an hour's interval into the same dog. It may be seen that the action of these doses is altogether equal to the sum of the effects which each had separately.

The results of three experiments carried out with ½ a unit of insulin (Burroughs, Wellcome and Co.) per kg. and 5 and 10 mg. of ephedrine on two dogs are shown in Fig. 10.

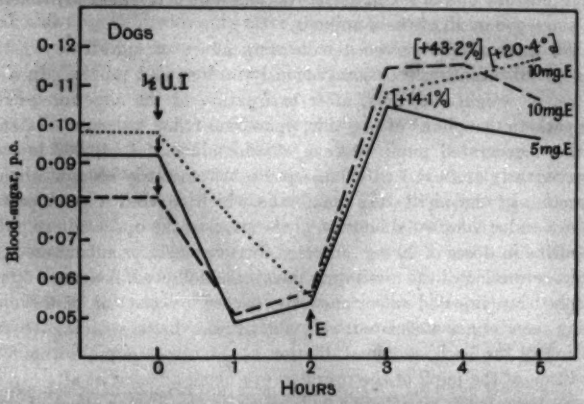

Fig. 10. Antagonistic action between ½ a unit of insulin (Burroughs, Wellcome and Co.) per kg. injected subcutaneously and 5–10 mg. of ephedrine per kg. introduced intravenously into the dogs. The solid and dotted lines refer to the same dog.

This shows that in accordance with the greater effect of ephedrine in producing hyperglycæmia in dogs, as compared with rabbits, the effects of insulin are more readily countered by ephedrine in the dog than in the rabbit.

NOTE ON ACTION OF EPHEDRINE ON THE DENERVATED PUPIL.

As was shown by Meltzer(21), about 24 hours after removal of the superior cervical ganglion in rabbits intravenous injection or a single instillation of adrenaline is capable of causing a maximal dilatation of

the pupil which may last for several hours. It was found later that the same holds true for the cat when the injection was given about 48 hours after excision of the ganglion. Instillation, however, gave no reliable results in the cat.

According to Chen and Schmidt(9) the pupils of dogs, rabbits and cats were dilated by ephedrine, whether the drug was injected intravenously or applied to the conjunctival sac. The mydriatic effect was wholly peripheral, as indicated by its limitation to the side to which the drug was applied. After degeneration of the long ciliary nerves, following extirpation of the superior cervical sympathetic ganglion of one side in rabbits, ephedrine caused definite dilatation of the denervated pupil in every one of six animals. Cocaine failed to cause mydriasis of the same eye in all of these animals.

We have also observed the dilating effect of ephedrine whether applied subcutaneously or intravenously in dogs and rabbits. In a cat 1·5 kg. of weight, however, after extirpation of the superior cervical sympathetic ganglion[1] of one side, ephedrine failed to cause dilatation of the degenerated pupil. 0·3 c.c. of adrenaline of 1 : 10,000 injected intravenously or 1 c.c. 1 : 1000 injected subcutaneously brought about a retraction of the nictitating membrane which covered a good part of the eye and a complete dilatation of the pupil of the operated eye. After ephedrine in doses of 20 mg., injected intravenously or subcutaneously, only a retraction of the nictitating membrane followed. A dose of 50 mg. of ephedrine, injected subcutaneously, caused a retraction of the nictitating membrane, associated with micturition, hairs standing on end, arching of the back, maximal dilation of the intact pupil, without the condition of the pupil of the operated eye being affected at all.

We have made no attempt to investigate the dilator effect of ephedrine on the intact pupil, but it would appear possible that it may be of central origin. Comparable perhaps with the varying action of ephedrine on the pupil in different animals may be the differences which exist also among human races. According to Chen and Poth(22) ephedrine brings about a definite dilatation of the pupil in the white races, but the action in coloured individuals such as Chinese or negroes is either very insignificant or absent.

[1] The operation of the removal of superior cervical sympathetic ganglion was performed by Prof. C. Lovatt Evans.

SUMMARY.

1. Ephedrine hydrochloride and ephetonine in doses from 25 to 30 mg. per kg. increase the blood-sugar content in rabbits by about 26–28 p.c. An increase of blood-sugar level to above 0·385 per 100 c.c. may be brought about by a dose of 80 mg. per kg., which is fatal.

2. Tyramine in doses ranging from 5 to 100 mg. per kg. has no appreciable hyperglycæmic action in rabbits.

3. Isoamylamine in doses of 25 mg. per kg. injected intravenously elevates the blood-sugar level 20 p.c. This however may be a reflex action, due to the irritant effect of this substance.

4. The antagonistic action of ephedrine for insulin in rabbits is very weak and may be shown only after very small doses of insulin.

5. Ephedrine hydrochloride injected intravenously in doses from 5 to 20 mg. per kg., increases the blood-sugar concentration in dogs by 65–75 p.c.

6. There exists a synergistic hyperglycæmic action between small doses of adrenaline and of ephedrine in dogs.

7. The anti-insulin action of ephedrine is accordingly more easily demonstrated in dogs than in rabbits. The hypoglycæmia brought about by insulin in dogs is very easily overcome even by small doses of ephedrine.

8. Though Chen and Schmidt reported that ephedrine caused dilatation of the pupil of a rabbit after excision of the superior cervical sympathetic ganglion, we find that ephedrine does not affect the denervated pupil in the cat.

We desire to express our thanks to Prof. C. Lovatt Evans for his suggestions, criticism and help given to us throughout the whole course of this research.

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SEX DIFFERENCES IN THE CHOLESTEROL CONTENT OF TISSUES[1].

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It has been known for a considerable time that variations in blood cholesterol occur during pregnancy, and it was not entirely unexpected therefore when the sex differences in the cholesterol content of tissues to be described here were demonstrated.

These sex differences were discovered during the examination of a large number of normal rabbits (8), when it was desired to have estimations of the cholesterol content of various tissues for purposes of control.

Before including both sexes in the series of controls, it was considered advisable to determine whether or not any material differences existed between the two sexes. Experiments soon showed that such differences did exist, and doe rabbits were discarded for control purposes, but the differences noted were deemed worthy of further investigation.

The results of this investigation are set forth here, together with the influence of pregnancy on the differences described.

Experimental. The animals used were rabbits and the tissues examined included suprarenals, ovaries, spleen, kidney, liver and brain. The procedure and method of estimation of cholesterol were similar to those previously used and described (8), but it must be emphasized again that with the method used only gross variations (over 20 p.c.) have been regarded as significant.

Results. These may be considered under the headings of the different organs and tissues, and the details are set forth in tabular form.

All figures refer to "total cholesterol," *i.e.* to free and combined cholesterol estimated together. This, admittedly, is not so satisfactory as having separate estimations of the free and ester cholesterol, but nevertheless the results obtained are significant both in themselves and as affording guidance to more detailed study, and the simplicity of the procedure has enabled a much larger number of experiments to be made than would otherwise have been possible.

[1] Work commenced during the tenure of a Beit Memorial Fellowship and completed under a grant from the Medical Research Council.

Suprarenals. The most striking contrasts were found with the adrenal glands where the average percentage of cholesterol was 12·3 in eight non-pregnant does, against 7·4 in 20 bucks, a difference of as much as 66 p.c.

TABLE I. Sex differences in the cholesterol content of the suprarenals.

Rabbit no.	Weight g.	Weight of suprarenals	p.c. cholesterol	Cholesterol ratio*	Duration of pregnancy (days)
		Buck rabbits.			
86	1500	0·270	11·1	20	—
45	1520	0·220	11·1	16	—
40	1710	0·570	10·7	36	—
30	2500	0·330	10·0	13	—
131	2080	0·420	9·9	24	—
122	1800	0·480	8·4	23	—
48	1380	0·230	7·8	13	—
143	1820	0·430	7·0	16	—
33	1970	0·420	6·8	14	—
44	1860	0·452	6·6	16	—
29	1645	0·210	6·3	8	—
35	1320	0·480	6·0	22	—
38	1800	0·330	6·0	11	—
73	1820	0·380	6·0	13	—
85	1440	0·160	5·9	6	—
54	1890	0·322	5·0	8	—
57	1700	0·498	4·1	12	—
95	1670	0·350	3·9	8	—
		Average	7·4	16	
		Normal doe rabbits.			
163	1820	0·18	20·0	20	—
124	2000	0·41	14·5	29	—
127	2480	0·29	12·9	15	—
114	2450	0·52	12·0	25	—
151	1700	0·36	12·0	25	—
118	2170	0·93	11·5	49	—
121	2100	0·31	10·2	15	—
164	1930	0·31	9·9	16	—
157	1860	0·31	7·7	13	—
		Average	12·3	23	
		Pregnant does.			
172	2220	0·27	13·2	16	17
153	2400	0·25	12·5	13	19
169	2650	0·46	12·2	21	14
158	1910	0·18	10·4	9	11
155	2900	1·22	9·2	38	28
161	2250	0·33	8·7	13	6
170	2130	0·23	7·9	8·5	22
159	2260	0·30	6·9	9	7
149	1660	0·295	6·4	12	Unknown
152	1940	0·53	5·7	15	28
		Average	9·3	14	

* "Cholesterol ratio" = actual amount in mg. of cholesterol in both suprarenals divided by the body weight of the animal in kg.

Moreover, the total amount of cholesterol in these glands was equally increased in the females, for the glands were of average weight, so that the alteration in cholesterol percentage could not be due to any alteration in weight of the glands.

It was found that in does the average "cholesterol ratio" or amount in mg. of cholesterol found in the two suprarenals divided by the body weight in kg. was 23, whilst in bucks this figure was 16. The excess of cholesterol in the suprarenals of the doe rabbits as compared with those of the bucks is shown in Table I.

During pregnancy this remarkable sex difference in the "cholesterol ratio" disappeared; the average value for this ratio in the ten pregnant animals examined is less than the average value in males, only two of the animals showing figures that are higher than this average and only one reaching or exceeding the average for non-pregnant does.

It was not possible, however, to establish any definite relationship between these changes and the period of gestation.

In two lactating animals the percentage of cholesterol was 9 in one case and 14 in the other.

Ovaries. These organs were examined in five non-pregnant and ten pregnant animals. In all cases except one non-pregnant animal, the

TABLE II. Showing cholesterol content of ovaries in pregnant and non-pregnant animals.

Rabbit no.	Body weight mg.	Weight of ovaries mg.	p.c. cholesterol
Pregnant animals.			
158	1910	0·68	5·5
152	1940	0·635	4·4
169	2650	0·94	4·3
170	2130	0·50	4·3
155	2900	0·95	4·1
172	2225	0·86	3·7
161	2250	0·95	3·3
149	1660	0·44	3·1
153	2400	0·975	3·1
159	2260	0·40	2·1
	Average	0·713	3·8
Non-pregnant animals.			
162	2120	0·345	6·8
151	1700	0·375	4·9
164	1930	0·29	4·5
157	1860	0·31	3·4
163	1820	0·10	Negligible quantity
	Average	0·284	3·9

amounts of cholesterol were very high; the values were higher than for any other tissue in the body except the suprarenals.

The reason for the exception in which only a negligible quantity of cholesterol was found, remains obscure. The average percentage of cholesterol did not differ materially in pregnant and non-pregnant animals, but it is to be observed that the average weight of the ovaries was much greater during pregnancy, so that the total amount of cholesterol is greater in the pregnant than the non-pregnant animal.

Other tissues. The average percentage of cholesterol in the spleen, liver, kidney and brain did not differ much in the two sexes. It may be noted, however, that with the spleen, liver, and brain the figures for non-pregnant animals are rather higher than those for pregnant animals, but the differences are not sufficiently marked to justify any deductions as to the significance of this.

TABLE III. Average amount of cholesterol (mg. per 100 g.) in the tissues of male, pregnant and non-pregnant rabbits.

	Spleen	Liver	Brain	Kidney
Buck rabbit	393	388	1800	437
Non-pregnant does	415	330	1940	400
Pregnant does	370	285	1800	—

DISCUSSION.

The physiology of cholesterol is so inadequately understood that the interpretation of the results recorded here can only be provisional. It seems probable that the large amount of cholesterol in the suprarenals of non-pregnant doe rabbits is a provision for pregnancy. The fact that in about half the cases the excess of cholesterol disappeared during gestation lends some support to this suggestion.

In accounting for these changes two possibilities must be considered. Firstly the suprarenals may merely store cholesterol and disgorge it during pregnancy, or alternatively the glands may work at a higher level of activity in the female than the male, the increased work being reflected in the increased amount of cholesterol present. In the latter case the special requirements of the animal may deplete the cholesterol supply during pregnancy, and the suprarenal cholesterol content will diminish to some extent. In a previous paper(6) support has been given to the theory of cholesterol synthesis rather than storage, and whilst the findings described here could be interpreted on either basis, it seems improbable that glands so small as the suprarenals could be adequate as a storage depot, whilst as a site for synthesis of cholesterol they might

form an important source of supply for the animal's needs during pregnancy. The double function of storage and synthesis is, of course, also feasible.

As far as the writer can ascertain the only previous observations made on the cholesterol content of the suprarenals in female animals were made during pregnancy by histological methods and revealed an excess (Albrecht and Weltmann(1)). Histological methods cannot, however, be accepted as showing the amounts of cholesterol present in these glands, and no contrasts have been made between the glands of non-pregnant and pregnant animals.

An increased supply of cholesterol is probably required during pregnancy, partly, as Baumann and Holly(2) suggest in their work on blood cholesterol, for the building up of extra maternal tissues such as the uterus, placenta, mammary glands and so forth. But another possible outlet must be considered, namely, to the fœtus for use in the growth of all its tissues, but particularly the central nervous system which is very rich in cholesterol. It remains, however, to be shown whether or not cholesterol can pass through the placenta for this purpose.

It is of importance to note that whilst most authors (Hermann and Neumann 1912(3), Chauffard, Laroche and Grigaut 1920(4), Benda 1923(5), Gardner and Gainsborough 1929(6)) record an increase in blood cholesterol in pregnant women, Baumann and Holly(7) find a decrease in rabbits, and this they hold to be usual for the lower animals, suggesting that the increase in man is due to an intoxication occurring during pregnancy, which does not occur in the lower animals.

It is interesting to find, therefore, that the decrease of blood cholesterol which occurs in pregnant rabbits is accompanied usually by a decrease in suprarenal cholesterol, and the results bear out the frequent statement that blood cholesterol generally runs parallel with suprarenal cholesterol, though, as the writer has shown, by increasing the amount of cholesterol in the blood by intravenous injection the amount in the suprarenal is not increased but even diminished(8).

Of two experiments made on lactating animals one showed a suprarenal cholesterol content intermediate between pregnant and non-pregnant values, suggesting that the cholesterol was gradually increasing from the lower values of pregnancy to the usual non-pregnant values. This was to be expected as the animal was examined only two days after parturition, whereas in the second animal, examined four days after parturition, a non-pregnant figure of 14 p.c. was obtained.

The large amount of cholesterol which the ovaries contain suggest that these bodies may also serve as a depot for cholesterol, to meet the increased demands of pregnancy.

Summary.

1. The suprarenal glands of non-pregnant doe rabbits were found to be much richer in cholesterol than those of buck animals.

2. During pregnancy this difference disappears.

It is suggested that cholesterol is prepared in the suprarenals of doe rabbits in greater amounts than in the buck animal, in order to meet the special requirements of pregnancy, such as building up extra maternal tissues and possibly for use by the fœtus.

3. The ovaries were found to be very rich in cholesterol, and may act as a supplementary source of cholesterol for the purposes mentioned.

4. The variations in the male, pregnant and non-pregnant female, of the cholesterol content of the liver, spleen, kidney, and brain were not sufficiently large to allow of conclusions being drawn as to their significance.

My thanks are due to Prof. W. Ramsden in whose Department this work has been carried out, and who has constantly given me most valuable advice and help.

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THE SOURCE OF EXPIRED CO_2 IN DECAPITATED, EVISCERATED CATS.

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The importance of measuring the products of metabolism by examination of the expired air led Kilborn(7) to determine the respiratory exchange of the decapitated eviscerated cat. He showed, in agreement with Burn and Dale(1), that after evisceration the respiratory quotient (R.Q.) rose to a value about 1, but concluded that this did not necessarily imply a change to carbohydrate metabolism. In fact, the amount of CO_2 expired seemed to depend upon the combined effects of (1) acidosis developing from increasing lactic acid concentration in the liverless animal, and (2) the relative overventilation caused by maintaining artificial respiration at a rate proper for the decapitated but otherwise intact animal. That overventilation and acidosis were concerned appeared from the fall in blood CO_2 and the opposite rise in lactic acid (Kilborn, Soskin and Thomas(8)).

Kilborn concluded that there was no evidence for a change in metabolism from the R.Q. determinations, but that by the acidosis and overventilation the acid-base equilibrium was so upset as to release CO_2 from the reserves of the body. The amount of CO_2 obtainable from disturbance of the familiar blood system could only account for a small part of the extra CO_2 expelled. In fact the large amount of extra CO_2 found, if it was assumed that the R.Q. really did not change on removal of the liver, suggested a labile reserve in the other tissues sufficient to affect respiratory measurements in all animals with changing acid-base equilibrium.

The extent of the tissue reserves for carbon dioxide has been studied by several investigators. Krogh(10) (p. 19) showed that overventilation of rabbits raised the R.Q. On the assumption that no change in the type of real metabolism occurred, it was indicated that 250 c.c. of extra CO_2 were blown off during 1½ hours. Kilborn showed that overventilation

raised the R.Q. of cats, and that the amount of CO_2 blown off by overventilation corresponded with the extra CO_2 derived from eviscerated cats. Conversely, CO_2 may be retained by animals breathing CO_2-rich mixtures, and in considerable amounts. Shaw(11) found that over 700 c.c. of CO_2 would be retained by cats weighing 3 kg. if they breathed air containing 7·5 p.c. CO_2. When the partition of the retained CO_2 between blood and tissues was calculated, the blood could only be held accountable for about 10 p.c. of the total amount absorbed.

The fact that the R.Q. may rise above unity after evisceration and during overventilation, and fall to 0 or a negative value during ventilation with CO_2-rich mixtures implies that it is the tissue-CO_2 reserves which are affected and not true metabolism. The first reserve tissue to consider is blood, in which the CO_2 content may be rapidly diminished or increased. The total amount of CO_2 in all of the blood of a cat is not very great, so that both Shaw and Kilborn show that it has no great importance as a reserve of CO_2.

It has been considered that muscles would enter largely into the CO_2 reserve system of the body, calculating their part principally by indirect methods. These inferential methods depend upon less accurate data than those of Shaw and Kilborn, and so cannot be used to explain their results. In an ingenious attempt to determine the influence of muscle on the alkali reserve of acidified perfused blood, Katz and Banus(6) could find no evidence of such influence when they perfused the isolated muscle. But since, on the contrary, they could reduce the acidity of perfused blood, by perfusing the whole leg, they concluded that their experiments showed that either bone or connective tissue could take up acid. On the other hand, Fenn(2) showed that the CO_2 content of frog muscle varied with the tension of CO_2 in the surrounding atmosphere.

All tissues are no doubt sooner or later affected by the acid-base balance of the diet. With regard specifically to bone the following data are available: the CO_2 content increases during growth (Wildt(14)), and may be increased in rickets and decreased in places of primary calcification (Howland, Marriott, Kramer(5), Kramer and Shear(9)). During a short period of acid feeding Goto(4) found a small decrease in the CO_2 content of rabbit bones. None of these data bears directly on the question whether the carbonate of the bones is directly available to meet the physiological demands in compensation for brief overventilation, but they show its lability.

These considerations led us to repeat the experiments of Kilborn,

examining the blood, muscle and bone as possible sources of the extra CO_2 obtained from eviscerated cats. We have therefore repeated Kilborn's experiments to see (1) what tissues contain sufficient CO_2 to serve as source of any considerable amount, and (2) if their CO_2 is available. Some of the preliminary experiments were performed with Dr Kilborn, and we have enjoyed the valuable advice of Prof. Macleod.

A first experiment showed that the total CO_2 of a mouse is great in comparison with the amount in blood, and the amount found, if applicable to the cat, offered a store of CO_2 which would not be greatly depleted by the loss of some 300 c.c. CO_2 per kg.

A mouse was eviscerated, skinned and placed in a tube with acid so that it could be subjected to diminished pressure by a mercury bulb. Analysis of the evacuated gases showed a CO_2 content of 2·8 c.c. per g. Converting this figure to the weight of an eviscerated cat gives the total CO_2 content of a cat weighing 2 kg. as 5690 c.c. In contrast to such a quantity of CO_2 the loss of a few hundred c.c. found by Kilborn does not seem serious.

As it is impracticable to determine the total CO_2 of a whole cat, we can by determination of the weight of various tissues and their average CO_2 content reconstruct the probable amount in the whole animal. Table I indicates the average weight of muscle and bone in percentage

TABLE I. Average amount of muscle and bone in the cat (2 animals).

	p.c. of the whole body wt.	p.c. of the decapitated and eviscerated body wt.
Muscle	44	60
Bone	25	24
Blood*	5·5	5†

* Meek and Gasser, 1918.

† 5 p.c. is only a guess, but compared with muscle and bone the possible error is insignificant.

of body weight in two cats from which the tissues were dissected and weighed separately, showing that muscle, bone and blood make up about 90 p.c. of the weight of the eviscerated, decapitated preparation. Later

TABLE II. CO_2 content of cat bones, including marrow. Taken during amytal anaesthesia.

Bone	Number of determinations	CO_2 in c.c. per g.
Tibia—whole	7	11·13
Tibia—epiphysis	1	7·57
Tibia—shaft	2	13·33
Tibia—distal part	9	12·71
Fibula—whole	12	13·66

tables will show that there is considerable variation in the CO_2 content of bones and muscles, but the average figures, shown in Table II for bone and Table III for muscle, give figures to go on.

TABLE III. The CO_2 changes in muscle and blood after evisceration and the expired CO_2.

No. of exp.	Time after evisc. in hrs.	Total c.c. CO_2 in 100 g. Muscle (av.)		Total c.c. CO_2 in 100 c.c. blood	
		Before evisc.	At end of exp.	Before evisc.	At end of exp.
1	3½	23·2	21·5	41·1	14·6
2	5½	25·1	13·9	41·1	22·0
3	3½	24·4	20·8	43·1	27·8
4	4½	18·9	17·5	28·2	14·5
5	3½	20·7	15·6	36·2	25·2
6	3½	16·1	13·0	29·6	11·8
7	3½	25·3	16·5	37·2	24·8

No. of exp.	Ventilation rate c.c. (at 22°) per kg. per min.	R.Q.		Extra CO_2 calculated: in c.c. per kg. body wt.	CO_2 lost from blood and muscle: in c.c. per kg. body wt.
		Before evisc.	After evisc. (av.)		
1	320	0·76	1·02	310	25
2	280	0·81	1·04	282	75
3	340	0·72	1·15	342	32
4	470	0·99	1·23	184	15
5	—	—	—	—	32
6	382	0·71	0·84	96	28
7	317	0·79	0·81	15	59

For muscle 0·22 c.c. per g. is evidently a fair figure, for bone 11 c.c. per g., and for blood 40 volumes p.c. Together these make in the eviscerated cat, per kg.

			c.c.
Total CO_2 in muscle	...	...	$0·22 \times 600 = 132$
Total CO_2 in bone	...	...	$11 \times 250 = 2750$
Total CO_2 in blood	...	...	$0·4 \times 50 = 20$
		Total	2902

Or in a preparation weighing 2 kg., 5804 c.c., not far from the 5690 c.c. inferred from the mouse.

The eviscerated, decapitated cat is, apart from skin, lungs and heart, exclusively a blood, muscle and bone preparation. The skin, lungs and heart were found to constitute about 10 p.c. of the eviscerated, decapitated weight, so that if its CO_2 content is taken as that of blood, muscle and bone, it will not be far short of the total. The average CO_2 content of bone, blood and muscle combined is 2902 c.c. per kg., and is therefore quite adequate to supply the 230 c.c. per kg. which Kilborn believed was expelled beyond the ordinary products of metabolism.

Having shown that the preparation contains so much CO_2 that less than 8 p.c. of it would supply the amount believed to have been expelled by alteration of the acid-base equilibrium—apart from metabolism—we must next look to see if these reserves are available. Accordingly the decapitated eviscerated cat preparation was examined for tissue CO_2 changes, observing the products of respiration as well.

Preparation.

The cats, under ether anæsthesia, were decapitated and maintained by artificial respiration, using the pump prepared by Mr F. L. Robinson. Samples of expired air were taken half an hour or more afterwards. Blood samples were also taken from the carotid artery, and the muscles used, usually Peroneus longus, Extensor digitorum and Tibialis anticus, were quickly dissected from one leg and kept in liquid air until determinations could be made. The bones were removed last from the same leg.

After the preliminary or normal air, blood, muscle and bone samples were taken, the animal was eviscerated. The cœliac and mesenteric vessels were tied off, also the portal vein and hepatic artery. After ligating the œsophagus and posterior end of the bowel, the viscera were removed, leaving the liver in place. As Burn and Dale (1) observed, the remaining liver does not appreciably participate in the circulation. The kidneys were not disturbed. In order to maintain some distention of the abdominal walls the cavity was packed with gauze moistened with saline.

Beginning immediately after evisceration the inspired air was passed through a meter, and frequent samples collected for analysis. The samples, of about 5 l. each, were collected in rubber bladders, from which about 50 c.c. lots were run immediately into Brodie gas pipettes. In this way they were kept in glass under mercury seals until the analysis.

Usually a blood sample was taken again during the experiment, and a final sample at the end. Finally, the muscles and bones were removed from the opposite leg.

Animals with low blood-pressure did not survive the period of about 3 hours necessary for results. A pressure of about 45 mm. mercury seems to be the minimum for maintaining active circulation to the hind limbs. Where blood-pressure was not measured, its effectiveness could be observed in the circulation of the hind legs at the termination of the experiment. All experiments are reported in which the circulation remained good and no technical difficulties interfered.

Analytical procedure.

The determinations of muscle CO_2 were made by a manometric method based on Van Slyke's apparatus for blood gases (Ferguson and Irving(3)). Blood CO_2 was determined by the usual procedure of Van Slyke and Neill(13). Samples of expired air were analyzed in a Haldane type apparatus. Gas volumes are corrected to standard conditions, and the oxygen consumption is calculated with proper reference to the nitrogen content of expired and inspired air. The determination of bone CO_2 was carried out by boiling in a closed flask with acid and drawing the CO_2 evolved through weighed absorption tubes. The method was checked against sodium carbonate solutions of appropriate CO_2 content, which were in turn checked by titration and by analysis in the Van Slyke apparatus.

Two typical experiments are summarized as Exps. 1 and 2. In the first, the R.Q. rose from the value of 0·76 before evisceration to 0·95 for

Experiment 1.

Weight: 3530 g.
Weight (eviscerated): 3005 g.

Respiration.

Volume per minute at 22°: 963 c.c.
R.Q. before evisceration: 0·76.
R.Q. after evisceration—at about 45 min. intervals: 0·95, 0·99, 1·07, 1·01, 1·14.

CO_2 expired in 3½ hr. after evisceration, as measured (0° and 760 mm.)	3650 c.c.
CO_2 calculated from oxygen consumption after evisceration and from R.Q. 0·76	2710 c.c.
Extra CO_2 expired	940 c.c.

Blood.

Total CO_2 in c.c. per 100 c.c.: initial 41·1; final 14·6.
Extra CO_2 from blood: $0{\cdot}05 \times 3005 \times 0{\cdot}265 = 39{\cdot}8$ c.c.

Muscle.

Total CO_2 in c.c. per 100 g.:

	Initial	Final
Peroneus l.	23·7	21·3
Extensor d.	22·7	21·2
Tibialis a.	—	22·0

Extra CO_2 from muscle: $0{\cdot}60 \times 3005 \times 0{\cdot}02 = 36{\cdot}0$ c.c.
Total extra CO_2 from blood and muscle: 75·8 c.c.

the first 38 minutes after evisceration, remaining close to 1 for 3½ hours, when the animal was killed. The blood-pressure was not measured, but the heart beat was regular and the peripheral circulation in the muscles persisted to the end. As in Kilborn's experiments, the rate of respiration used for the decapitated animal was continued for the eviscerated. The results also quite agree with his, and we have calculated the extra

CO_2 on the same basis, assuming that the initial R.Q. persists. 940 c.c. more CO_2 were expired than would have been produced by metabolism at the original quotient. As to the source of the extra CO_2, less than 10 p.c. has been derived from blood and muscle, leaving 865 c.c. still to be accounted for.

Exp. 2 shows results for a much stronger cat, in which the blood-pressure and circulation remained satisfactory for 5 hours and 10 minutes,

Experiment 2.

Weight: 3000 g.
Weight (eviscerated): 2520 g.

RESPIRATION.

Volume per minute at 22°: 706 c.c.
R.Q. before evisceration: 0·81.
R.Q. after evisceration: 0·97, 0·96, 1·14, 1·21, 0·93.

CO_2 expired in 5 hr. 10 min. after evisceration, as measured (0° and 760 mm.)	3300 c.c.
CO_2 calculated from O_2 consumption after evisceration and from R.Q. 0·81	2580 c.c.
Extra CO_2 expired	720 c.c.

BLOOD.

Total CO_2 in c.c. per 100 c.c.: initial 41·1; final 22·0.
Extra CO_2 from blood: $0{\cdot}05 \times 2520 \times 0{\cdot}191 = 24{\cdot}1$ c.c.

MUSCLE.

Total CO_2 in c.c. per 100 g.

	Initial	After 2 hr. 10 min.	After 5 hr. 10 min.
Peroneus l.	24·4	17·8	—
Latissimus d.	25·8	18·4	—
Deltoideus	—	—	12·9
Triceps	—	—	14·8

Extra CO_2 from muscle: $0{\cdot}60 \times 2520 \times 0{\cdot}11 = 166$ c.c.
Total extra CO_2 from blood and muscle: 190 c.c.

when the animal was killed. The initial rate of ventilation, 706 c.c. per minute, is greater than for the heavier cat in Exp. 1, and the oxygen consumption (Table IV) is only about half as great. Muscle CO_2 was determined at two intervals after evisceration, and shows that the loss of muscle CO_2 is progressive. In order to follow the rate of muscle CO_2 changes, the Latissimus dorsi, Deltoideus and Triceps were examined. These three are all at or below the heart level and centrally situated where circulation would be more easily maintained. There is no indication in this cat that the muscles behave differently, but the cat kept an exceptionally strong heart action through the experiment. It is quite likely that in some experiments the circulation in the peripheral muscles is not so good as in the deeper, and these latter may in consequence lose more CO_2. The total CO_2 lost by blood and muscles represents about

30 p.c. of the total calculated CO_2 loss. In this experiment, then, the contribution of muscle to the CO_2 in the expired air is quite significant.

Seven experiments summarized in Table III show that after evisceration the R.Q. rises, but the average R.Q. of the eviscerated animal is not always unity. The rise in R.Q. is as obvious in Exp. 4, although starting at 0·99. The cat had not been fed before the experiment, and it appears from the low initial CO_2 of muscles and blood that CO_2 was already being blown off before evisceration. Evidently a similar situation prevailed in Exp. 6 diminishing the amount of extra CO_2 lost.

There were also a large number of experiments in which some of the determinations were not made. These partial experiments agree, as far as they go, in showing the rise in respiratory quotient after evisceration toward or well above unity. As source of the extra CO_2, which ranges from about 100 to 350 c.c. per kg. body weight, blood and muscle changes would usually account for from 10 to 30 p.c.

It is only in Exp. 2 that the decline in muscle and blood CO_2 content is parallel, about 50 p.c. in each case. The animal maintained a vigorous circulation for a long period, and the result probably represents the best state of equilibrium between blood and muscle in any experiment. The excess CO_2 expired is still not entirely to be assigned to changes in blood and muscle. Even if all of the muscle CO_2 were lost it would only amount to 380 c.c., so that no chance error in selecting the muscles could furnish much of the 207 c.c. per kg. not accounted for by loss from blood and muscle.

The reason for the expulsion of CO_2 from the blood and muscles, according to Kilborn(7), is to be found in the relative overventilation and acidosis. In our experiments we have set the initial ventilation of the decapitated cats at a rate as low as appeared consistent with maintenance, and then after evisceration kept the rate unchanged. Table IV

TABLE IV. O_2 consumption before and after evisceration.

No. of experiment	Body weight in g.		Before	After	
	Whole	Eviscerated	c.c. per min.	First sample c.c. per min.	Last sample c.c. per min.
1	3530	3005	47	21	15
2	3000	2520	20	13	10
3	2950	2430	35	11	9
4	2620	2165	13	4	8
6	2800	2350	17	10	8
7	3350	2875	17	10	13

shows that the oxygen consumption declines at evisceration, but remains approximately constant afterwards, so that an adequately ventilated

decapitated cat receives after evisceration about twice the necessary ventilation. Kilborn, Soskin and Thomas(8) likewise reported considerable increases in blood lactic acid, supporting the view that acidosis is an important contributing factor. Our observations confirm their views. In addition, several determinations of muscle lactic acid showed slight increases after evisceration.

In only one experiment, Exp. 7, is the CO_2 lost by blood and muscle adequate to account for the extra CO_2 calculated on the basis of a steady R.Q. In the usual experiment it seemed natural to look to the bones, as the holders of most of the body CO_2, as possible sources. In six experiments reported in Table V the CO_2 content of bone was determined. In

TABLE V. CO_2 content of bones. Eviscerated cats, at conclusion of experiment.

No. of exp.	Bone	Hours after evisc.	Before evisceration: CO_2 c.c. per g.	Before evisceration: $\frac{Ca}{CO_2}$	After evisceration: CO_2 c.c. per g.	After evisceration: $\frac{Ca}{CO_2}$	Change c.c. per kg. body wt.
8	Tibia—shaft	3	13·8	—	13·5	—	−75
9	Tibia—whole	3½	9·87	—	9·92	—	+13
10	Femur—distal half	3½	9·97	—	9·72	—	−63
5	Tibia—distal	3½	14·32	7·50	13·81	7·45	−135
	Fibula	—	15·73	7·12	15·16	7·38	
6	Tibia—distal	3½	11·82	—	11·60	—	−82
	Fibula	—	13·51	7·85	13·05	8·16	
7	Tibia—distal	3½	12·21	7·30	11·89	7·33	−67
	Fibula	—	13·61	7·67	13·39	8·00	

all but one case the bone CO_2 was perceptibly diminished after evisceration. The changes are all small, but usually a little greater than the average difference found in the control determinations shown in Table VI.

TABLE VI. CO_2 content of normal bones (including marrow).

No. of exp.	Bone	Right: c.c. CO_2 per g.	Left: c.c. CO_2 per g.	Difference
11	Tibia—whole	11·70	11·64	0·06
12	Tibia—whole	12·55	12·70	0·15
13	Tibia—proximal epiphysis	7·50	7·64	0·14
	Tibia—shaft	12·72	13·00	0·28

From Table VI it is apparent that there is a difference in the CO_2 content of epiphysis and shaft which might cause a large error where part of a bone, as in the case of the tibia, is sampled. It has been shown that the ratio $\frac{Ca}{CO_2}$ for bones generally in an individual is remarkably constant (Howland, Marriott, Kramer(5), Kramer and Shear(12)), Accordingly total calcium determinations were made on five of the bones examined,

using the method of Clark and Collip on samples from the acid residue of the CO_2 determinations. Three of these show the $\frac{Ca}{CO_3}$ ratio increase about equivalent to the CO_2 loss, one shows only an insignificant increase, and one an insignificant loss.

If from the loss of CO_2 by the bones examined we may infer a similar loss by all of the bones, then the total amount indicated is quite significant. In the last column of Table V the CO_2 lost from bones is calculated on the basis of 250 g. of bone per kg. body weight losing CO_2 at the rate indicated. It is evident that the amount lost is near that of the extra CO_2 expired which had previously not been explained by blood and muscle CO_2 losses.

There is certainly no exact agreement between the amount of CO_2 lost from bone and the unexplained CO_2 expired, nor if there were would it have any greater significance, on account of the accumulated uncertainties of the various determinations. It is plain, however, that muscle and blood do not contain enough CO_2 to explain half of the usual extra CO_2 loss, and that a loss of bone CO_2 amounting to from 3 to 6 p.c. would account for the remainder. A difference of such magnitude is on the border of that detectable with the errors in determination and natural variation of controls, although these usually fell below 4 p.c. and were in either direction.

The CO_2 content of bones is subject to reduction in prolonged disturbances of metabolism. Goto(4) found that administration of acid-forming substances to rabbits produced after a number of days a small decrease in bone CO_2 content, which we have been able to confirm. In rickets changes in CO_2 content have been observed (Howland, Marriott, Kramer(5), Kramer and Shear(12)), as well as changes during growth. Inasmuch as there is evidence for the lability of bone carbonates in prolonged metabolic disturbance, it is reasonable to suppose that the carbonates could lose CO_2 during an acute alteration of the acid-base equilibrium.

If the bone carbonates are rapidly sensitive to alterations in the acid-base equilibrium and easily take part in its maintenance by giving off CO_2, then the converse should also be true. In experiments such as those of Shaw(11), where cats are ventilated with air containing 7 p.c. CO_2, considerable quantities of CO_2 are at first retained. Eventually the cat resumes expiration of CO_2 so that its R.Q. returns to that of the start of the experiment. In the process of readjustment the cat retains several hundred c.c. of CO_2, of which only about 12 p.c. is retained by blood. In repeating Shaw's experiments we have found that the CO_2

absorbed by tissues may be much more than that given off by eviscerated animals. The increase in muscle CO_2 is greater than the loss shown by eviscerated cats, and the unaccounted-for remainder absorbed is also greater. But in these experiments, which will be reported later, we have been unable to prove a consistent increase in bone CO_2.

In our examination of the idea that the bone carbonates may participate in the rapid regulation of acid-base equilibrium, we are not satisfied with the evidence we have obtained. If the bones of the eviscerated cat may lose CO_2, then absorption should appear in the converse type of experiment. We therefore feel that it is not proved that bones serve for immediate relief of disturbance of the acid-base equilibrium. On the other hand, the extra CO_2 expired can never be entirely accounted for as originating in blood and muscle, for the total amount in them, even if it is all available, is not sufficient.

The extra CO_2 expired by eviscerated cats is apparently not produced by an alteration in true or metabolic R.Q. In the first place, the fact that some must be attributed to loss from blood and muscle throws the whole amount in doubt. Overventilation of the entire animal produces similar amounts of extra CO_2, and there is in that case no reason for supposing a change in the metabolic R.Q. Underventilation or breathing high CO_2 mixtures oppositely affects the R.Q., which returns later to the original value. From the combination of evidence, direct and indirect, the expired CO_2 is a particularly bad indicator of the kind of metabolism in the eviscerated cat.

It is worth remark that our experiments differ from those of Burn and Dale in one important respect. Their eviscerated cats were plentifully supplied with sugar during the experiment. Under their conditions, the accentuation of carbohydrate metabolism is certainly favoured, so that until we can examine the CO_2 content of tissues in eviscerated cats perfused with sugar we certainly cannot criticize their findings. On the other hand, the idea that the muscles of the eviscerated cat are restricted to carbohydrate metabolism, which has been often asserted by others on the strength of Burn and Dale's experiments, is not justified. Since we could not account for all of the extra CO_2 by showing its source, we should perhaps be careful about saying that there is no change in metabolism, but it is equally obvious that the change is partly an artefact. Evidently control of the acid-base equilibrium in the eviscerated cat is so complicated as to make very difficult the estimation of the true metabolism by measuring the products of respiration.

SUMMARY.

The apparent R.Q. of the decapitated cat, with artificial respiration, rises after evisceration to a value of one or above.

There is no evidence that the true R.Q. of metabolism changes. If no change occurs, considerable amounts of extra CO_2 are expired from changes in the acid-base equilibrium.

The CO_2 content of blood is reduced—nearly two-thirds in some cases. The absolute amount contributed to the extra CO_2 is relatively insignificant.

The CO_2 content of the muscles may be reduced by as much as two-fifths. Combining the loss from blood and muscle only accounts for between 11 and 57 p.c. of the extra CO_2.

There are good reasons for expecting that bone might participate in the rapid regulation of acid-base equilibrium by contributing part of the extra CO_2. Losses in bone CO_2 are indicated by analysis, in amounts similar to the part unexplained by blood and muscle changes. The smallness of these analytical differences, and the failure of bone to show gains when animals are retaining CO_2, prevent acceptance of the bone as the proved source of the unexplained extra CO_2.

The R.Q. of eviscerated cats represents a profound disturbance of the acid-base equilibrium, and can hardly be interpreted to indicate a change in metabolism.

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ON THE PARTIAL PRESSURES OF OXYGEN AND CARBON DIOXIDE IN ARTERIAL BLOOD AND ALVEOLAR AIR.

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Ever since the work of Haldane and Priestley(1) (1905) on the pressure of CO_2 in alveolar air, and that of Krogh(2) (1910) on the mechanism of gas exchange in the lung, it has been generally believed that the partial pressure of CO_2 is the same (to a close approximation) in arterial blood and alveolar air. The relation, however, of the partial pressure of oxygen in alveolar air to that in the arterial blood has not been determined with the same accuracy. This uncertainty is due in part to the dispute concerning the rôle played by the lungs in oxygen transfer, in part to difference of opinion regarding the mechanics of pulmonary ventilation, and also to lack of precise knowledge of the facts. It is the purpose of this paper to present data with reference to gas exchange in the lung. Our experiments have been performed on normal men while they were breathing air or low oxygen mixtures.

Method.

The modification of the Haldane-Priestley method used in obtaining samples of alveolar air has been previously reported(3). For the present work we have estimated the partial pressures of both CO_2 and O_2 in the same samples of air. In the above-mentioned communication the method used for the determination of arterial CO_2 pressure was described. At that time, however, equilibration of the blood was carried out at 37·5° C. In the present series of experiments all equilibrations were done at the oral temperature of the subject. Since the temperatures found under basal conditions for normal subjects are in general at least one degree below the standard temperature, the earlier determinations are about one millimetre higher than the present determinations, owing to the effect of temperature on the CO_2 dissociation curve. A small error in the earlier work is due to neglect of the effect of acid formation in

blood after its removal from the body. We have now attempted to eliminate this error by determining the magnitude of the acid effect in the blood of each subject by the following procedure. For each case, 10 c.c. of venous blood were equilibrated, immediately after bleeding, for periods of 90 to 120 minutes at 37° C., and $pCO_2 = 40$ mm. Samples of 1 c.c. were removed from the tonometer at intervals of 10 to 30 minutes and the CO_2 content determined. Since the rate of decrease in CO_2 content was found to be a linear function of time, extrapolation to $t = 0$ by means of the line drawn through the determined points should give the true bicarbonate level of the blood in the body. For the sake of convenience the data are plotted as in Fig. 1. They indicate that the rate of

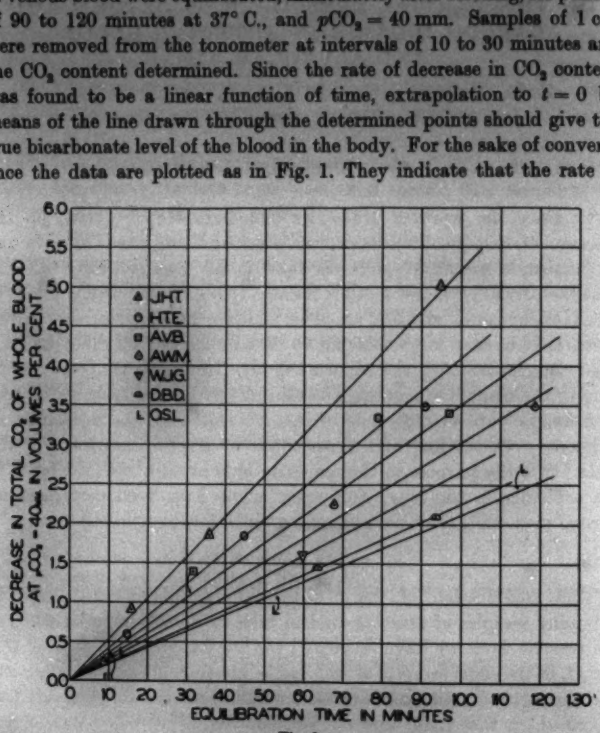

Fig. 1.

acid formation varies from subject to subject. The average loss of bicarbonate for all of our subjects was about 0·4 vol. p.c. during equilibration. For each case the level of the fully oxygenated CO_2 dissociation curve was corrected for the acid change as thus determined. The curve was also corrected to the level corresponding to the p.c. oxyhæmoglobin found in arterial blood. This is a simple correction owing to

the linear relation between the bicarbonate content of blood and the oxygenation of hæmoglobin at constant pressure of carbon dioxide.

In previous experiments it has been customary to refer the experimentally determined saturation of arterial blood to a standard oxygen dissociation curve in order to estimate the partial pressure of oxygen. In our present experiments we have tried to avoid the small errors involved in such a procedure, which by cumulation may become large, by (1) determining two or three points on the oxygen dissociation curve in each experiment, (2) adjusting the carbon dioxide pressure of equilibration near that of blood, and (3) correcting the points to the precise value for carbon dioxide pressure of arterial blood. To make this correction, typical oxygen dissociation curves of a normal individual at carbon dioxide pressures of 20 and 40 mm. were plotted logarithmically with $\log pO_2$ as a function of $\log \frac{Hb}{HbO_2}$. We then assume that, even though the position of these two curves may vary from case to case, the effect of small changes in carbon dioxide pressure upon percentage saturation is the same for all normal individuals, and further that the effect is linear. Many observations in this laboratory indicate that the first assumption is correct, and that the second assumption involves at most an error of about 1 p.c. in the corrected value for saturation when the difference in carbon dioxide pressures is 10 mm. This is a greater difference than any that has been involved in our corrections. The data are given in Table III.

Arterial blood was used for all points determined on the CO_2 and O_2 dissociation curves, the determinations being made with the van Slyke-Neill manometric apparatus. To insure complete saturation of the blood for oxygen capacity determinations, it was equilibrated in tonometers containing air for $\frac{1}{2}$ hour or longer at 20° C.

Low oxygen mixtures for breathing were obtained by diluting outside air with nitrogen in a spirometer of 600 litres capacity. The duration of experiments when such mixtures were breathed was 35 minutes or longer. The periods for which the total ventilations are recorded in Table III were begun with the 25th minute, each determination representing a period of 10 minutes. Arterial puncture under novocaine anæsthesia was done immediately after this collection period. The subjects were all in basal condition, and were studied in the prone position.

The partial pressure of carbon dioxide.

In 1924 Bock and Field(3) published a series of 24 determinations of alveolar and arterial CO_2 pressures. The average of 21 of these showed a difference in pressure of not more than 0·5 mm. This result agreed closely with that of Krogh's study of rabbits with his aerotonometer method(2). Similar data in man were shortly obtained by Dautrebande(4), and later by Richards(5). Recently Grollman(6) has concluded that true alveolar CO_2 pressures cannot be obtained while the subject lies flat owing to the reduction in vital capacity and complemental air, characteristic of the prone position. However, no experimental data are given in support of this conclusion. We now present another set of determinations, recorded in Tables I and II. With one

TABLE I. Carbon dioxide dissociation curves.

Subject	Date	Oral temp. °C.	pCO_2 mm. Hg	O_2 saturation p.c.	Total CO_2 content, whole blood: Uncorrected vol. p.c.	Total CO_2 content, whole blood: Corrected vol. p.c.
D. B. H.	7 xi. 28	36·8	36·0	100	48·35	49·35
			59·0	100	57·95	58·95
					57·60	58·60
H. T. E.	7 xi. 28	36·9	34·0	100	43·1	44·0
			39·4	—	46·1	47·0
J. H. T.	7 xi. 28	36·8	34·6	100	44·5	46·3
			61·2	100	56·8	58·6
					56·65	58·45
A. W. M.	9 xi. 28	36·8	29·5	100	43·3	44·1
					43·4	44·2
			56·1	100	55·3	56·1
O. S. L.	9 xi. 28	36·8	28·0	100	42·15	42·75
					42·4	43·0
			53·6	100	54·6	55·2
					55·1	55·7
W. J. G.	9 xi. 28	37·1	33·9	100	44·95	45·60
					45·30	45·95
			67·5	100	59·1	59·75
					59·1	59·75
D. B. D.	26 ii. 29	36·5	35·0	100	48·9	50·05
			67·5	100	61·0	62·15
					61·2	62·35
W. J. G.	12 iii. 29	36·2	35·6	100	46·8	47·8
					46·95	47·95
			60·2	100	57·3	58·3
					57·3	58·3

TABLE I. Carbon dioxide dissociation curves (*contd.*).

Subject	Date	Oral temp. °C.	pCO_2 mm. Hg.	O_2 saturation p.c.	Total CO_2 content, whole blood: Uncorrected vol. p.c.	Total CO_2 content, whole blood: Corrected vol. p.c.
H. T. E.	13 iii. 29	—	44·2	100	48·0	49·3
					48·0	49·3
			41·3	100	46·9	48·2
					46·9	48·2
J. H. T.	20 iii. 29	36·3	28·2	100	40·5	43·4
					40·55	43·45
			61·2	100	56·6	59·5
					56·6	59·5
A. W. M.	22 iii. 29	—	33·3	100	43·6	44·4
			77·2	100	61·05	61·9
A. W. M.	22 iii. 29	—	36·0	92·5	44·9	47·3
			36·1	85·4	45·2	47·2
			57·0	91·3	54·85	57·2
D. B. D.	27 iii. 29	36·4	41·5	93·7	52·2	52·35
					52·1	52·45
			59·5	100	58·8	59·5
					58·65	59·35
D. B. D.	27 iii. 29	36·6	35·4	75·5	49·9	49·4
					49·9	49·4
			62·8	62·6	62·65	61·35
					62·45	61·25
A. V. B.	29 iii. 29	36·1	38·8	96·6	49·6	50·0
			61·0	100	57·8	58·4
A. V. B.	29 iii. 29	36·0	35·9	88	47·5	48·1
			51·1	80	57·8	58·0
A. V. B.	29 iii. 29	36·3	30·6	70	44·9	44·6
A. V. B.	3 iv. 29	97·2	42·0	97·4	49·5	50·1
A. V. B.	3 iv. 29	97·2	22·3	98·4	39·2	39·8

TABLE II. Comparison of the partial pressure of carbon dioxide in alveolar air and arterial blood.

Subject	Date	Oxygen in inspired air p.c.	Total CO_2 content arterial blood vol. p.c.	Arterial pCO_2 corrected mm. Hg	Alveolar pCO_2 mm. Hg	Δ mm. Hg
D. B. D.	7 xi. 28	20·9	53·0	44·0	44·9	−0·4
					42·9	
					43·0	
H. T. E.	7 xi. 28	20·9	47·8	40·6	41·3	+0·5
			48·0		41·6	
					40·6	

TABLE II. Comparison of the partial pressure of carbon dioxide in alveolar air and arterial blood (*contd.*).

Subject	Date	Oxygen in inspired air p.c.	Total CO_2 content arterial blood vol. p.c.	Arterial pCO_2 corrected mm. Hg	Alveolar pCO_2 mm. Hg	Δ mm. Hg
J. H. T.	7 xi. 28	20·9	49·25 49·50	41·0	41·0 41·0 40·3 40·2	−0·4
A. W. M.	9 xi. 28	20·9	50·2 50·0	41·3	43·4 44·1 44·1	+2·6
O. S. L.	9 xi. 28	20·9	50·2 50·15 50·3	42·2	41·9 41·7 41·3	−0·5
W. J. G.	9 xi. 28	20·9	49·3	41·0	42·1 39·3 40·1	−0·5
D. B. D.	26 ii. 29	14·1	52·8 52·65	40·7	41·2 40·5	+0·1
W. J. G.	12 iii. 29	14·1	50·4 50·45	40·9	39·8 37·6 39·6	−1·9
H. T. E.	13 iii. 29	13·3	47·3 47·2	39·5	38·3 38·3	−1·2
J. H. T.	20 iii. 29	9·6	44·2 44·6	29·5	30·4 29·0 28·3 27·8	−0·7
A. W. M.	22 iii. 29	20·9	49·4	43·7	43·5 43·5 43·8	−0·1
A. W. M.	22 iii. 29	10·4	47·6	34·6	36·2 36·1	+1·5
D. B. D.	27 iii. 29	20·9	53·0	43·0	42·8 42·0 44·3	0·0
D. B. D.	27 iii. 29	10·4	42·4 42·9	23·4	22·5 21·7 21·2	−1·6
A. V. B.	29 iii. 29	20·9	50·64	40·2	40·0 40·7 40·6	+0·2
A. V. B.	29 iii. 29	13·6	49·55	38·8	36·9 37·2 35·2 35·4	−2·4
A. V. B.	29 iii. 29	10·5	45·3	32·2	29·2 30·8 30·4	−2·1
A. V. B.	3 iv. 29	24·7	49·55 49·55	40·5	36·6 36·2	−4·1
A. V. B.*	3 iv. 29	20·9	42·90 42·85	26·8	23·5 20·8	−4·6

* Hyperpnœa, etc.

or two exceptions all experiments done while the subjects breathed outside air show a close correspondence (mean difference 0·2 mm.) of the pressures for CO_2 in alveolar air and arterial blood. This close approximation to equilibrium is presumably due to the rapid rate of diffusion of CO_2. The data further substantiate the usefulness of alveolar CO_2 determinations in normal subjects as a measure of the partial pressure of CO_2 in arterial blood. We do not wish to imply that the pressure of alveolar CO_2 is constant during the respiratory cycle. Our experiments indicate that the pressure of CO_2 found, during the phase when the sample of alveolar air is taken, is approximately equal to that of arterial blood.

In the experiments when low oxygen mixtures were breathed there appears to be an excess pressure of CO_2 in arterial blood (mean difference 1·0 mm.) over that present in alveolar air. More experiments should be done to determine whether or not this second order difference is a real one.

The partial pressure of oxygen.

According to Haldane, the partial pressure of oxygen, as estimated from samples of alveolar air, does not represent the average pressure of oxygen to which the mixed arterial blood is exposed in the capillaries of the lung. He writes as follows: "It is evident that the relation between blood supply and ventilation in individual groups of alveoli is not an even one. In some alveoli the oxygen runs down and CO_2 accumulates faster than in others. Hence in some the blood is less perfectly oxygenated....Hence the oxygen percentage of the mixed alveolar air becomes altogether deceptive as an index of the degree of oxygenation of the mixed arterial blood...." (7). The fan-like expansion of the lung described by Keith (8) supports this theory.

A solution of the problem may be sought from a comparison of pressures of oxygen found in alveolar air and in arterial blood under varying pressure heads of oxygen in the lung. Our experimental data on the subjects are included in Tables III and IV. The arterial oxygen

TABLE III. Oxygen dissociation curves.

			Observations on equilibrated blood				
Subject	Date	HbO_2 capacity vol. p.c.	pCO_2 mm. Hg	pO_2 mm. Hg	Total O_2 content	HbO_2 content	O_2 saturation
D. B. D.	26 ii. 29	17·92 17·87	39·2	58·8	16·51 16·35	16·28	91·0
			40·2	77·2	16·95 17·05	16·81	94·0
			40·2	98·6	17·80 17·70	17·45	97·5

TABLE III. Oxygen dissociation curves (*contd.*).

			Observations on equilibrated blood				
Subject	Date	HbO_2 capacity vol. p.c.	pCO_2 mm. Hg	pO_2 mm. Hg	Total O_2 content	HbO_2 content	O_2 saturation
W. J. G.	12 iii. 29	21·30 21·28	40·5	26·2	11·60	11·54	54·2
			41·8	51·3	18·59 18·52	18·43	86·6
			41·5	77·7	20·69	20·50	96·3
H. T. E.	13 iii. 29	21·03 21·10	39·3	23·5	9·70 10·23	9·64 10·17	46·1 48·4
			39·5	49·2	17·36 17·25	17·20	81·7
			38·1	73·8	19·60	19·42	92·3
J. H. T.	20 iii. 29	21·99 21·96	36·6	20·3	5·82	5·77	26·2
			36·5	46·7	18·60 18·60	18·48	84·1
			35·5	66·6	20·86	20·69	94·1
A. W. M.	22 iii. 29	21·03 20·98	43·7	55·6	18·79	18·65	88·8
			39·2	92·2	20·51	20·28	96·7
A. W. M.	22 iii. 29	21·05 21·05	35·8	30·2	12·56	12·48	59·3
			36·1	48·8	18·09	17·97	85·4
			57·0	75·2	19·40	19·21	91·3
			36·0	72·3	19·65	19·47	92·5
D. B. D.	27 iii. 29	18·63 18·57	40·6	37·4	13·94	13·85	74·5
			43·1	62·8	17·03	16·87	90·7
			41·5	75·6	17·62	17·43	93·7
D. B. D.	27 iii. 29	19·26 19·26	35·1	22·7	8·73	8·67	45·0
			35·4	29·0	14·61	14·54	75·5
			35·6	52·0	17·50 17·45	17·35	90·1
A. V. B.	29 iii. 29	21·30 21·40	40·4	54·7	19·87	19·75	92·6
			38·8	79·6	20·71	20·57	96·1
A. V. B.	29 iii. 29	21·92 22·10	35·9	48·9	19·45	19·35	87·9
			36·3	68·7	20·73	20·48	93·1
A. V. B.	29 iii. 29	22·42	30·6	30·3	15·70	15·62	69·6
			31·7	50·7	20·20	20·08	89·5
A. V. B.	3 iv. 29	20·35 20·35	42·0	121	20·12	19·82	97·4
			40·1	86·6	19·90 19·74	19·68 19·52	96·7 95·9
A. V. B.	3 iv. 29	20·62 20·62	22·3	129·4	20·62	20·30	98·4
			21·5	74·1	20·17	19·99	96·9

TABLE IV. Comparison of the partial pressure of oxygen in alveolar air and arterial blood.

Subject	Date	Pulse rate	Respiratory rate	Ventilation l per min.	Oxygen in inspired air p.c.	O_2 saturation arterial blood p.c.	Arterial pO_2 mm. Hg	Alveolar pO_2 mm. Hg	Δ mm. Hg.
D. B. D.	26 ii. 29	60	12	5·4	14·14	85·0	53	55	+2
W. J. G.	12 iii. 29	66	16	5·3	14·09	89·0	55	55 61 58	+3
H. T. E.	13 iii. 29	83	12	6·8	13·32	85·3	55	50 50	−5
J. H. T.	20 iii. 29	96	15	9·3	9·58	64·7	30	31 34 35 36	+4
A. W. M.	22 iii. 29	70	11	5·1	20·93	92·2	63	90 95 94	+30
A. W. M.	22 iii. 29	94	15	7·3	10·42	57·8	29	32 31 31	+2
D. B. D.	27 iii. 29	56	10	5·1	20·93	94·4	77	93 97 95	+18
D. B. D.	27 iii. 29	71	12	9·2	10·42	90·2	45	53 55 55	+9
A. V. B.	29 iii. 29	61	6	4·7	20·93	95·3	78	106 104 100 101	+25
A. V. B.	29 iii. 29	65	6	6·3	13·57	84·0	45	56 58 56 56	+12
A. V. B.	29 iii. 29	75	6	8·2	10·54	81·5	40	47 43 43	+4
A. V. B.	3 iv. 29	58	7	4·8	24·68	95·3	83	139 139	+56
A. V. B.*	3 iv. 29	—	30	—	20·93	96·0	75	136 138	+62

* Hyperncea produced by a respiratory rate of 30 per minute, beginning one minute before arterial puncture and continuing during puncture.

pressures range from 78 mm. down to 29 mm. and the oxygen saturations from 96 p.c. to 57·8 p.c. The alveolar oxygen pressures vary from 139 mm. to 31 mm. As a check on the accuracy of the arterial oxygen

pressure determinations Fig. 2. was constructed. This figure shows (1) the upper portion of the oxygen dissociation curves for the blood of

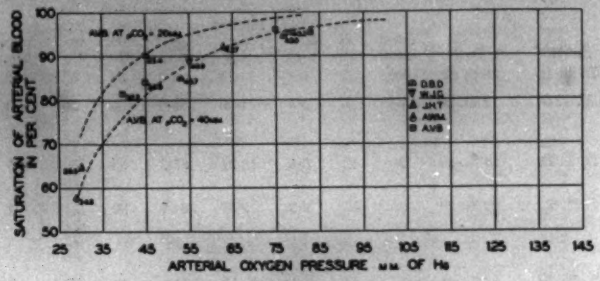

Fig. 2.

A. V. B. at carbon dioxide pressures of 20 mm. and 40 mm. and (2) points experimentally determined for the specimens of blood employed in this investigation. The coordinates of these points give the values of oxygen saturation and oxygen pressure. Beside each point the value of carbon dioxide pressure is printed. The source of the blood is also designated. Inspection of the figure shows that nearly all the points determined fall quite closely in their expected position with reference to normal oxygen dissociation curves. If the alveolar oxygen pressures are plotted against the corresponding arterial oxygen saturations and

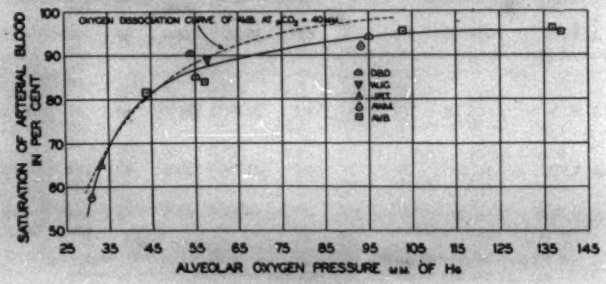

Fig. 3.

a free-hand curve drawn through the points, the result is seen in Fig. 3 in which the curve is compared with a normal oxygen dissociation curve.

The last two experiments recorded in Table IV were performed to see if a large pressure head of oxygen in the lung, produced by different methods, would exert an appreciable effect upon the saturation of arterial blood in the lung. In each instance the arterial saturation was not sensibly changed, even though the pressure difference between the lung and the blood withdrawn from the artery reached approximately 60 mm. These experiments suggest the question whether the saturation of arterial blood in the lung can be raised above about 96 p.c. In one of seven of our experiments during exercise performed two years ago(9) and in the experiments of Himwich and Barr(10), during heavy exercise, saturations of the blood with oxygen were found above 96 p.c. Small experimental errors may, however, account for such figures. In view of the present data, obtained after long practice in the use of the methods, these older determinations are at least open to question.

In the final experiment recorded in Table IV the subject breathed outside air, but increased his respiratory rate to 30 per minute, the breathing being kept as deep as possible at this rate. The alveolar CO_2 pressure fell to 22 mm., and meanwhile the O_2 pressure rose to 137 mm. The saturation of arterial blood was 96 p.c. In the subject A. V. B., therefore, oxygenation of the blood seems to be equally effective at his normal respiratory rate of six per minute, with an alveolar oxygen pressure of 103 mm., and when the rate of respiration is 30, and the oxygen pressure in the lung is raised to 137 mm.

The mechanism of oxygen exchange in the lung.

In the last column of Table IV we have recorded the difference in the pressure of oxygen between alveolar air and arterial blood. Reference to Fig. 4, in which these pressure differences are plotted against the oxygen saturations of the arterial blood, reveals a physiological relation of more than passing interest. Granting that the data are not numerous it is doubtful if a greater number of experiments would alter the general character of the results. In five experiments the pressure of oxygen in the lung exceeds that of the arterial blood by 4 mm. or less. In only one of these does the saturation of the blood exceed 85 p.c. Above this level of saturation the pressure of oxygen in the lungs rises rapidly, and the curve becomes very steep in the region of high oxygen saturation.

In seeking an explanation of these facts, we believe that the data concerning the movement of carbonic acid from blood to alveolar air should be carefully considered. As stated above, in every one of the present experiments a close approximation to equality of carbon dioxide

pressure in arterial blood and samples of alveolar air collected by our present method has been experimentally demonstrated, and this approximation is closest when the oxygen pressure of the inspired air is

Fig. 4.

normal. Therefore, it seems probable that in general all the blood while passing through the lung reaches a condition at least very close to equilibrium with the alveolar air that we find by our method, in respect of carbon dioxide pressure, and that the number of alveoli in which this condition is not established must be very small indeed, or else that such alveoli must be temporarily supplied with very little venous blood.

The second observation to be accounted for is that when low oxygen mixtures are breathed the pressure of oxygen in arterial blood differs from that of the same alveolar air that is in carbon dioxide equilibrium with the same blood by no more than 3 or 4 mm. In view of the well-known fact that for equal pressure gradients oxygen is much less diffusible than carbonic acid, it seems probable, therefore, that under these circumstances the ventilation of the alveoli which are being supplied

with venous blood is also sufficient for the movement of oxygen. It is to be regarded as sufficient because under the conditions of these experiments the pressure of dissolved oxygen in the blood at the surface of the red cell when it leaves the lung must differ very little from its pressure in alveolar air. Then what is the explanation of the large differences between oxygen pressure in alveolar air and arterial blood when normal air is breathed? For this condition both rate and volume of breathing are slightly lower than when the inspired air is rarified. It cannot, therefore, be positively denied that the ventilation of certain portions of the lung may in this manner become inadequate. But it seems probable that no great change of this kind can occur, and our observation that forced breathing of normal air produces no measurable increase of the oxygen content of arterial blood seems to be an argument against the importance of such a factor.

Thus we are disposed to turn in another direction for an explanation of the facts and to suggest the following working hypothesis. In normal man the ventilation of the physiologically active lung alveoli and the nature of the diffusing membranes are such that the difference in oxygen pressure on the two sides of the capillary wall at the point where the blood passes out of contact with the inflowing oxygen is but a few millimetres. But the blood, because it is heterogeneous and because mixing in the interior of the red cells cannot be effective, requires perhaps several seconds to attain a state of equilibrium. Ordinarily the results of this condition are not always manifest. But when the hæmoglobin is nearly completely oxygenated the unoxygenated molecules will be, in general, deep in the interior of the red cells, and this may modify the rate of diffusion of oxygen at that point. In any case, however, equal differences in oxygenation are associated with small differences of oxygen pressure when oxygenation is not nearly complete, with large differences of oxygen pressure when oxygenation is nearly complete. Thus the final stages of oxygen diffusion, taking place after the blood is cut off from further oxygen supply, should produce little change in oxygen pressure at the surface of the red cell when the final stage of oxygenation is less than, say, 80–90 p.c., and large differences if the final stage lies above that amount. This conclusion, thus roughly stated, appears to be a consequence of the shape of the oxygen dissociation curve.

In order to fix our ideas we may consider the simple problem of the mixing of equal volumes of two portions of blood in which equilibrium has been reached under such conditions that there exists a difference of 5 p.c. in the saturation of the hæmoglobin. If these saturations are

55 p.c. and 60 p.c. the oxygen pressures before mixing, for the blood of A. V. B., will be 28·0 mm. and 30·4 mm. respectively, and after mixing the pressure will be 29·2 mm. But, if the saturations are 93 p.c. and 98 p.c., the pressures before mixing will be 65·6 mm. and 108·0 mm., and after mixing the pressure will be 79·0 mm.

We suggest, therefore, that when normal air is breathed the pressure of oxygen at the surface of the red cell as it leaves the lung is some 20 mm. greater, the oxygenation of the hæmoglobin some 0·2 p.c. less than a few seconds later when equilibrium has been established. No doubt other possible explanations may be devised, and we are far from believing that it is yet possible to give a complete explanation of the complex conditions. For example, there appears to be a real difference between the two individuals, A. V. B. and D. B. D., as shown by the broken line of Fig. 4. For this we have at present no explanation unless it be related to the low "diffusion coefficient" or to the slow breathing of the subject A. V. B. Our preference for the suggested hypothesis rests upon the belief that the present investigation yields at least a strong presumption that the diffusion process across the capillary wall of the lung is not the principal cause of the difference of 20 mm. or more between the oxygen pressures of alveolar air and samples of arterial blood when ordinary air is breathed, and the belief that a homogeneous chemical reaction between hæmoglobin and oxygen, if stirring is adequate, should reach a state of equilibrium at 96 p.c. oxygenation no less rapidly that at 50 p.c. oxygenation.

Summary.

Carbon dioxide partial pressures in alveolar air and arterial blood are found to be approximately equal, while oxygen partial pressures are lower in arterial blood than in alveolar air. This difference is larger when oxygen partial pressure is high, far smaller when oxygen partial pressure falls below 40–60 mm. Hg. It is suggested that these observations may be explained by the hypothesis that oxygen equilibrium is not attained until after passage through the lung capillaries.

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THE ACTIVITY OF THE SPLEEN DURING ANAPHYLACTIC SHOCK.

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Since current opinion considers the spleen as an organ which may regulate the circulating blood volume by dilatation or constriction through the activity of the smooth muscles of its capsule and trabeculæ (1, 2, 3), the behaviour of the spleen during the phenomenon of anaphylaxis promises some interesting results. Four cats were accordingly sensitized to human serum, the dosage being 1 c.c. subcutaneously given. After a sensitizing period of two weeks, two of the animals were studied under urethane anæsthesia by the standard kymograph methods, recording respiration, carotid pressure, and splenic volume as measured by a splenic oncometer.

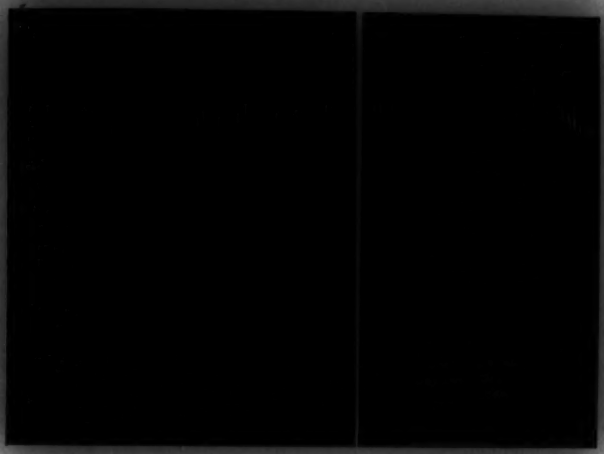
While marked excursion of the lever of the oncometric tambour was obtained by vagal stimulation, representing a decrease in volume of about 0·5 c.c., neither dilatation nor constriction occurred upon the injection of the provocative dose of human serum. That the cat was sensitized is shown by the marked fall in blood-pressure and by the respiratory embarrassment. This result is of extreme interest, since the volume of the spleen ordinarily follows passively the blood-pressure. These results are shown clearly in Fig. 1, *A* and *B*.

The third and fourth cats were prepared for kymograph records of blood-pressure and respiration. The abdomen was opened, the splenic vessels were tied off and the spleen removed. The provocative doses of serum produced the same effect as on the intact animal, showing that sensitization had taken place.

The removed spleens of the last two animals were tested *in vitro*. The spleen of the third cat was mounted in a muscle warmer, capacity 100 c.c., bathed with Tyrode solution at 34° C., and irrigated with oxygen. The introduction of 0·5 c.c. of human serum failed to produce any activity, although a loop of isolated intestine, removed before the cat was shocked, showed marked contractions under the same conditions.

The spleen of the fourth cat was sharply lacerated before removal.

Hæmorrhage was not marked and there was no fall in blood-pressure. The removed spleen was then mounted as described for the previous one.



A *B*

Fig. 1. Cat. 2 kg.; female. Sensitized March 20, with 1 c.c. human serum, subcutaneously given. Tracings of respiration, splenic volume and blood-pressure, April 3. *A*. Showing the contraction of the spleen when the left vagus was stimulated; both vagi intact, coil at 10 cm. *B*. Showing the inactivity of the spleen during the fall of blood-pressure due to the intravenous injection of a provocative dose of 1 c.c. of human serum.

The introduction of the provocative dose of human serum again failed to produce an effect, although 2 c.c. of 1 : 10,000 solution of adrenaline produced a marked and continuous contraction (Fig. 2).

The failure of the spleen to react in anaphylaxis both *in vivo* and *in vitro* is consistent with the results obtained by Barcroft, Harris, Orahovats and Weiss(1) with histamine, since it is generally thought that the active agent in anaphylaxis is a histamine-like product.

In a series of investigations on the effect of various drugs on the excised spleen of the hog and the calf, pilocarpine and atropine in large doses also failed to produce any effect, although adrenaline and barium chloride always produced contraction. This observation seems to call for a reclassification of these drugs as parasympathetic or for a further study of the innervation of the spleen.

From these observations it would seem that the spleen is not stimulated to contract and throw its blood-content into the general circulation,

Fig. 2. Cat. 2·4 kg.; female. Sensitized April 24, with 1 c.c. human serum, subcutaneously given. Tracing showing the activity of the isolated spleen, suspended in Tyrode solution; 34° C., oxygen irrigation; first, when a provocative dose of human serum was introduced; and secondly, when adrenaline was added.

to compensate for the fall of blood-pressure caused by the provocative agent in anaphylaxis.

SUMMARY.

The spleen is entirely passive during anaphylaxis, both *in vivo* and *in vitro*.

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THE ACID-BASE EQUILIBRIUM IN FROG'S MUSCLE.

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It is now generally agreed that the main, perhaps the only, causal process in muscular activity is the conversion of glycogen, an undissociated substance, to highly dissociated lactic acid, and the consequent shift in acid-base equilibrium. At present it is difficult to say what happens in the first stage, the contraction process, but it seems clear that the relaxation process consists essentially of the neutralization of the lactic acid by the buffer substances in the whole tissue, as first suggested by Meyerhof(1). The buffer substances must be similar to those present in blood, though it is not possible to speak with the same certainty about them. It is uncertain what part is played by the proteins, and they as well as some of the non-protein constituents are very unstable. In blood all the constituents can be treated as being in solution, but in muscle some of the protein is not in solution at any time and more is readily thrown out of solution. The titration curve of a protein is altered according to whether it is dissolved or partially undissolved. The parts played by the different constituents must at present be left undetermined, but the tissue as a whole can be examined. Buffer curves of muscle have been obtained by Furusawa and Kerridge(2); Carlström, Ege and Henriques(3) and by Fenn(4). The first named, working on cat's skeletal and heart muscle, have shown that there is a rough correspondence between the change in hydrogen-ion concentration produced by adding lactic acid to minced muscle and that produced by the development of lactic acid in the intact tissue either by stimulation or *rigor mortis*. The errors of the method are necessarily large, so that it still remains to be seen whether the whole change in acid-base equilibrium is accurately accounted for by the gylcogen-lactic acid reaction. The experiments to be described were intended to examine this question as regards frog's skeletal muscle, by comparing the change in lactic acid content of the tissue with the change in acidic or basic groups as determined by titration.

First of all it will be well to consider certain other reactions that may

occur, or are known to occur, in muscle under anaerobic conditions and which involve the acid-base equilibrium. There are six of them.

1. Ammonia production has been found by various workers and especially studied by Parnas and Mozolovski(5). In frog's muscle the maximum ammonia under any condition is about 0·005 *N*, while the maximum lactic acid is ten times as much, viz. 0·05 *N*. By stimulation a relatively smaller quantity is produced. If the NH_3 all comes from the deaminization of adenosine phosphate as Parnas(6) considers, then the increase of base will be proportional to the amount of NH_3 formed, as the dissociation constants of adenosine and inosine phosphates are similar (Wassermeyer(7)). In any case the increase in ammonia is only about one-tenth of the lactic acid increase in frog's muscle.

2. There may be hydrolysis of phosphate esters which would produce a change in the direction of alkalinity, as inorganic phosphoric acid is a weaker acid than these organic derivatives. In frog's muscle the amount is very small. The maximum quantity of "lactacidogen" found by Embden and Jost(8) is equal to about 0·005 *M* hexose, so that changes in acid-base equilibrium due to breakdown of this substance will be negligible in comparison with lactic acid production.

3. Lohmann(9) considers that pyrophosphate may be produced in muscle or may change to orthophosphate under certain conditions. The changes in acid-base equilibrium from this cause may be complex, but again are of a very small order of magnitude compared with the lactic acid change.

4. Phosphagen, the compound of creatine and phosphoric acid, studied by the Eggletons and others (Eggleton and Eggleton(10)), by its breakdown would give a more alkaline reaction: the change being more marked at acid reactions than near neutrality (Fiske and Subarrow(11)). Stella(12) finds the equilibrium concentration of phosphate in the fluid surrounding resting muscle to be about 0·003 *M*; in fatigued muscle it is 0·006 *M*, a small change from the point of view of the present discussion. In rigor, however, the equilibrium concentration is 0·042 *M*, agreeing with the inorganic phosphate content of the tissue as found by chemical analysis. The change in rigor therefore is appreciable. It should be noted that apart from any genuine change in the tissue the great instability of phosphagen may lead to experimental error if the extent of breakdown due to handling the tissue varies. In the experiments to be described the phosphagen was probably completely broken down in all cases, so that any effect due to it would be eliminated. As far as these four factors are concerned there should be no appreciable change

as the result of stimulation, but in rigor an increase in base partly compensating for the increase in acid due to lactic acid. Of these factors (1) and (4) would seem to be quantitatively the most important.

There are two further possibilities.

5. Oxidative processes are not being considered and there is no functional change of CO_2 to consider, but as the tissue normally has a considerable CO_2 pressure, escape of CO_2 may lead to experimental errors (Fenn(4)).

6. Lastly, there is the possibility of protein changes, which will be considered again in discussing the results.

It should be possible to decide experimentally whether there is or is not any appreciable change in acid-base equilibrium, other than that due to lactic acid, by estimating lactic acid and simultaneously applying a titration method to the whole tissue, which will determine the total acid combining capacity or total base combining capacity. The results obtained by these methods are referred to for brevity and clarity, though not quite accurately, as the free base and acid of the tissue respectively.

The determination of free base is simple in theory and with certain limitations very satisfactory in practice. It consists in adding to the tissue a measured excess of trichloracetic acid, filtering, and titrating the excess acid in the filtrate from which the combined acid is calculated. By this method CO_2 is completely expelled and phosphagen completely broken down, so that these two factors are eliminated. The accuracy of the method is limited by the fact that a large excess of acid is needed to precipitate proteins so that what is measured is a small difference between two large quantities.

The method for the determination of acid is less satisfactory. It is essentially a modification of Sörensen's formol titration method. The tissue cannot be directly titrated with free alkali under any conditions because the reaction takes a considerable time, and in alkaline solution irreversible hydrolytic changes in proteins and other constituents occur. The method used therefore is to add excess of disodium phosphate in the presence of alcohol and formalin, filter and estimate the amount of acid phosphate formed and therefore the combined base by titrating the filtrate with caustic soda.

The experiments fall under two heads: those on the changes due to stimulation and those in which changes in the minced tissue were examined. The changes taking place in the minced tissue are apparently similar to but much more rapid than those taking place in natural *rigor*

mortis. Because development of *rigor mortis* in intact frog's muscle is inconveniently slow, well mixed minced muscle was used; in this way errors in sampling are at the same time minimized.

Experimental procedure.

Stimulated muscle, base and lactic acid. For experiments on stimulated muscle it was found convenient to use the legs of a single large *R. esculenta*. The animals, for which I have to thank Prof. Verzàr, were obtained from Hungary. The frog was cooled on ice, killed and the upper part of the body above the urostyle cut off along with the viscera. The legs with skin intact were packed in ice. The right-hand nerve trunks were cut, electrodes placed under the left-hand nerves and single make and break shocks nearly maximal in strength sent in. The switch in the primary circuit was opened and closed by hand and the rate of stimulation regulated so that there was always at least partial relaxation between stimuli. After about 200 shocks distinct signs of fatigue were visible and stimulation was stopped. The legs were then skinned, the muscles of the fatigued and resting leg dissected out separately and minced in small tissue mincers cooled on ice. 3–5 g. of the minced tissue were dropped at once into tared stoppered flasks containing about 15 c.c. of 0·2 *N* trichloracetic acid, the flasks being cooled on ice. The flasks were weighed to 0·01 g. as soon as they reached room temperature, and if necessary further trichloracetic acid added till there was 5 c.c. for each gram of tissue.

A further 3–5 g. from each leg were weighed in a tared stoppered flask and kept at 35° C. for 1½ hours. This treatment was found to give nearly maximum lactic acid production without much of the secondary changes mentioned later, and was intended for comparison between the two legs. 5 c.c. of 0·2 *N* trichloracetic acid were added for each gram of tissue. These flasks and the other two were then weighed to 0·01 g. to measure accurately the acid added, well shaken and left overnight.

The next morning the contents of each flask were filtered into two small tared stoppered flasks, the first fraction used to rinse the flasks, 4–8 c.c. of filtrate collected in each and weighed. Two drops of methyl red were added and the filtrate titrated with carbonate free soda of about 0·15 *N* strength to a colour corresponding to *p*H 5·6–5·8. The caustic soda was delivered from a micro-burette graduated to 0·01 c.c. with a fine jet delivering a drop of 0·01 c.c. About 0·05 c.c. is sufficient for the change from pale red to yellow, and the colour change due to much less than 0·01 c.c. is easily visible. In a few cases the filtrate was

titrated further to phenol phthalein. There is appreciable buffering due to muscle constituents, for trichloracetic acid itself gives practically the same titre with the two indicators, the difference however is the same in filtrate from "resting" and "rigor" muscle, so no appreciable error is likely from this cause. Two filtrates from the same sample very seldom differed by more than 0·2 $N/100$ (milligram-equivalents per 100 g. of tissue). The titre is not changed after standing under the acid for three days. The quantity of trichloracetic acid used is immaterial provided there is no protein in the filtrate.

The neutralized filtrates were then used for lactic acid estimation after treatment with copper sulphate and lime. This treatment not only cleans up the solution for the lactic acid estimation but has the advantage of giving a biuret reaction if there is protein in the filtrate, as happened in a few cases. In such a case the titre of the filtrate will be too high. To the neutralized filtrate 1 c.c. of saturated $CuSO_4$ solution and 5 c.c. of 7 p.c. milk of lime were added, and the flask weighed. After shaking and standing ½ hour the liquid was filtered into a small tared flask and a convenient amount taken for lactic acid estimation. This estimation was carried out, with minor modifications, by the steam distillation method described by Boyland(13) which is essentially a modification of Clausen's(14). A freshly prepared MnO_2 suspension was used for oxidation: at the beginning and end of each set of estimations the iodine solution was standardized in duplicate by means of a standard lithium lactate solution. A blank for the reagents was deducted. In some later experiments the oxidation was carried out in the presence of dilute phosphoric acid as recommended by Friedemann and Kendall(15). This was a distinct improvement, giving not only a slightly higher yield of acetaldehyde, but, what is more important, a more constant yield and a much more stable end point in the iodine titration.

All the results are calculated as milligram-equivalents per 100 g. of tissue ($N/100$).

For experiments on the change between "rest" and "rigor" the procedure was similar. Most experiments were done on *R. temporaria*. All leg muscles from three or more frogs, if necessary, were minced together, part being fixed at once in cold trichloracetic acid, the other part treated in various ways to bring about lactic acid production. The samples were left at different temperatures up to 35° C. and to some a drop of toluene was added. Toluene speeds up lactic acid production, but probably also speeds up the secondary changes which generally occurred to some extent more especially after long standing. For determination

of the free acid the tissue is fixed by treatment with alcohol, the procedure being in all other respects similar to that with trichloracetic acid previously described. 4–6 g. of tissue are taken and 4 c.c. of alcohol for each gram of tissue. The alcohol used was industrial spirit purified by distillation over caustic soda, 5 c.c. of 0·5 p.c. phenol phthalein added to each 100 c.c. It is preferable, but not necessary, to leave the tissue under alcohol for 24 hours to complete the coagulation of proteins. There was then added for each gram of tissue 4 c.c. of a solution containing 1 part of 40 p.c. formalin and 3 parts of 6 p.c. crystalline disodium phosphate. The whole is then weighed, well shaken and left overnight. A blank mixture of alcohol, formalin and phosphate is made up at the same time. The filtration and titration should be made between 12 and 18 hours after mixing, or at some other definite interval, as the titre does not remain unchanged.

Two lots of about 10 c.c. of filtrate are collected in small tared stoppered flasks, weighed and titrated with 0·15 *N* caustic soda. The filtrate is turbid and slightly coloured. The end point taken is where the first definite change in colour occurs. The addition of 0·03–0·04 c.c. more of caustic soda should produce a distinct but pale pink. Matching can generally be made to within 0·02 c.c. in spite of differences in colour and turbidity, if the first change be taken. This corresponds in aqueous phosphate solutions to *p*H 7·8–8·0. 10 c.c. portions of the blank are titrated similarly. Sodium phosphate frequently crystallizes out during the operation, but this makes no appreciable difference to the end point. The colour of the indicator remains constant in a stoppered flask for many hours. *O*-cresol phthalein does not appear to be any better for this purpose than phenol phthalein. Thymol phthalein is not satisfactory; it is not soluble enough, its colour change is too far on the alkaline side for maximum sensitivity and its blue colour renders it useless in turbid solutions, where a pink is easily seen. The method outlined above is probably capable of improvement. The conditions that must be fulfilled are (1) there should be a large excess of a buffer salt from which the tissue can take up base, (2) at the same time the final concentration of salt and the end point of titration should be such that the filtrate is not too highly buffered at that point, (3) the acid form of this buffer salt (NaH_2PO_4) must not be precipitated, (4) a steady state must be attained without secondary changes such as hydrolytic processes, (5) proteins should be completely precipitated. The last (5) is probably least important. (2) and (4) are perhaps incompatible, if there is sufficient available base there may always be secondary changes.

It is likely that violent shaking at a low temperature for a few hours would give better results than longer treatment at room temperature: this has not been tried as the simple method seemed sufficiently accurate for the purpose in view. A few experiments were made using sodium acetate as the buffer salt with small excess of caustic soda. This was much better than phosphate as regards (3), (4) and (5), but clearly failed as regards (1): there was not sufficient base available. Possibly the salt of an acid with a dissociation constant intermediate between the second one of phosphoric acid and that of acetic acid, or a mixture of salts would fulfil the requirements.

Results.

Stimulated muscle. The results obtained in the four most satisfactory experiments on *R. esculenta* in good condition are given in Table I.

Table I.

mg.-equivalents per 100 g. of tissue.

Experiment	Resting. Right leg. Base + lactic acid = A	Stimulated. Left leg. Base + lactic acid = B	Change $B-A$
1	24·0 + 0·6 = 24·6	22·5 + 1·5 = 24·0	−0·6
2	24·6 + 0·1 = 24·7	23·0 + 1·6 = 24·6	−0·1
3	22·3 + 0·2 = 22·5	21·6 + 1·4 = 23·0	+0·5
4	22·4 + 0·8 = 23·2	21·8 + 1·7 = 23·5	+0·3
		Mean change	0·0

Experiment	Rigor. Right leg. Base + lactic acid = C	Rigor. Left leg. Base + lactic acid = D	Change $C-A$	Change $D-B$
1	19·1 + 4·9 = 24·0	19·7 + 5·0 = 24·7	−0·6	+0·7
2	20·1 + 5·6 = 25·7	20·3 + 5·6 = 25·9	+1·0	+1·3
3	18·3 + 4·9 = 23·2	18·2 + 4·9 = 23·1	+0·7	+0·1
4	19·6 + 3·9 = 23·5	19·1 + 4·0 = 23·1	+0·3	−0·4
		Mean change		+0·4

In all cases except the last, about 200 single shocks were given. In the last the muscles were fatigued after 150, evidently owing to a higher initial lactic acid concentration. The rigor values are obtained from a second sample of the minced resting and stimulated muscles after 1½ hours at 35° C. If the only change in acid-base equilibrium is due to lactic acid the available base should diminish as the lactic acid increases, so that the sum remains constant. An increase in the sum indicates an increase in base, such as might be due, for instance, to ammonia formation. The experimental error in each determination is of the order of ± 0·2 N/100, so that it is clear that in stimulation any change other than lactic acid formation is no larger

than this. In "rigor" there is a small increase in base. The increase is hardly outside experimental error and is probably due to secondary changes, to be discussed shortly. The figures show also that the right and left legs of the same frog are comparable though there are considerable differences between different frogs. Four other experiments which are comparable with these are not included in the table, as being for various reasons less accurate. If, however, the mean change, stimulated-resting muscle, in all eight be taken, it comes to $-0{\cdot}04\ N/100$ which is still negligible in view of the greater experimental error.

As the change in lactic acid produced by stimulation in these experiments is not much greater than the experimental error, only a rough correspondence is shown by them. By pushing the stimulation to complete inexcitability more lactic acid could be produced, but only with an increased probability of secondary changes such as may be found in minced muscle.

Changes in minced muscle. In Table I it will be seen that on standing for 1½ hours at 35° C. there is in most cases an apparent increase in the

TABLE II.

mg.-equivalents per 100 g. of tissue.

Experiment	Lactic acid *A*	Base *B*	Acid *C*	*A* + *B*	*C* − *A*
1. Rest	0·5	25·3	9·0	25·8	8·5
Rigor	5·3	23·7	14·4	29·0	9·1
Difference	+4·8	−1·6	+5·4	+3·2	+0·6
2. Fatigue	1·2	24·2	8·0	25·4	6·8
Rigor	4·3	23·9	14·7	28·2	10·4
Difference	+3·1	−0·3	+6·7	+2·8	+3·6
3. Rest	0·5	25·6	8·7	26·1	8·2
Rigor	4·6	21·3	13·1	25·9	8·5
Difference	+4·1	−4·3	+4·4	−0·2	+0·3

sum base + lactic acid which is however hardly outside experimental error. In a previous series of experiments on summer frogs (*R. temporaria*) in good condition a similar result was obtained. Various procedures were used: in some cases toluene was added and the experiments were done at various temperatures and with various times of standing. In most cases there was a slight increase, generally largest where most lactic acid was found. The mean change was $+0{\cdot}6\ N/100$ (7 exps.). When experiments were done on winter frogs a much larger change was found in many cases which was clearly outside experimental error. A few results are given in Table II, in which the free acid as well as the basic groups have been determined.

These represent extreme cases; all the other results are intermediate.

Generally, either there is agreement between all three sets of determinations, as for (3), so that the sum base + lactic acid, or the difference acid − lactic acid, are constant or else if they disagree as in (1) and (2) the base + lactic acid increases while the difference acid − lactic acid increases also. In other words, if there is an increase in basic groups there is also an increase in acidic groups in excess of the lactic acid.

Taking the mean of seven experiments on winter frogs, the results are:

Excess base	...	...	...	1·3 *N*/100
Excess acid	...	...	...	0·6 *N*/100

According to the results of Parnas and Mozolovski(5) the production of ammonia is not likely to be as great as the excess of base found nor to be accompanied by an increase of acid. Of the other changes enumerated on p. 296 the only kind likely to be appreciable and to result in increase of acid is a hydrolytic change in proteins. That there is a change of this kind is shown by several independent pieces of evidence. In two cases where minced muscle had been standing for a long time to obtain full development of lactic acid, the trichloracetic acid filtrate gave a slight biuret reaction after the van Slyke copper-lime treatment. This was further investigated in the following manner.

Filtered aqueous extracts of frog's muscle were made, one at once with ice-cold water, the other from minced muscle which had stood with toluene for 1½ hours. To 5 c.c. quantities were added varying quantities of 0·2 *N* trichloracetic acid. In both cases 2 c.c. or more of the acid gave a precipitate which settled leaving a clear supernatant liquid, but whereas in the extract from "resting" muscle the filtrate after adding 2 c.c. of trichloracetic acid gave only a faint biuret test, that after adding 10 c.c. gave none; even with 10 c.c. of trichloracetic acid the filtrate from the "rigor" muscle gave a distinct biuret reaction. Similar results were obtained with rat muscle. With a sufficient excess of precipitant this more soluble fraction can be precipitated; it is small in amount, difficult to separate by filtration and has not been further investigated. It is noticeable that when resting and stimulated muscle is treated rapidly and in the cold with protein precipitants there is very little flocculent precipitate in the mixture, but that in muscle which has been allowed to stand or in muscle taken from an animal during or after rigor there is a considerable flocculent precipitate. In minced tissue or in rigor there is clearly a structural degradation going on which is accompanied by an increase in the fraction of the protein which is not readily precipitated by ordinary protein precipitants. It seems

probable therefore that the increase in amount of such protein is accompanied by the opening up of fresh acidic and basic groups, as the titration figures suggest. This change, however, must be essentially *post mortem* or associated with death changes and no part of the ordinary functional behaviour of the tissue. It may be concluded that, if these secondary changes and possible changes in phosphagen which would not be measured in these experiments are left out of account, there is no evidence for any other change in acid-base equilibrium greater than about 10 p.c. of the lactic acid produced.

SUMMARY.

By a titration method described in the text the change in acid combining capacity of frog's muscle produced by stimulation has been found to agree with the change in lactic acid. For the changes in minced tissue the agreement is not so close, apparently owing to secondary *post mortem* changes. As there is an excess both of basic and acidic groups under these conditions part at least of the secondary change is to be attributed to protein hydrolysis.

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STUDIES ON WATER DIURESIS.

Part II. The excretion of urine after hypophysectomy and decerebration.

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Introduction.

That a relation exists between the region of the floor of the third ventricle and urine secretion has long been established both from clinical and experimental evidence. Opinion differs, however, as to whether the polyuria which usually follows operative manipulation in this region is the result of a pathological disturbance of the pituitary body or of irritation or injury to some adjacent portion of the brain.

Dott(8) obtained polyuria by inserting platinum plates into the sella turcica and by sectioning the infundibulum, but observed no physiological disturbances resulting from complete ablation of the posterior lobe of the pituitary body, the completeness of the lesion being subsequently confirmed by microscopical examination of the base of the brain. He believed that polyuria is the result of a disturbance of the pars intermedia. Camus and Roussy(4), Hanchett(14), Curtis(5) and Roussay(18) found that injury to the tuber cinereum alone would cause a typical diabetes insipidus, whereas injury to, or extirpation of, the pituitary body did not alter the urine flow. Bourquin(3) and Bayley and Bremer(2) state that the mamillary bodies may also be involved. Recently Fulton and Bremer(12) found that, in cases of tumour of the region of the third ventricle, polyuria occurred when post-mortem examination indicated that the pituitary body was in no way involved. Finally an examination of various protocols on total and partial hypophysectomy indicates that total extirpation is by no means always followed by polyuria although removal of the anterior lobe may be associated with a profound diabetes insipidus(1, 6, 19).

In opposition to this view the antidiuretic action of extracts of the posterior lobe of the pituitary body in cases of diabetes insipidus, and

during normal water diuresis, is evidence, *ad hoc*, that the hypophysis is involved in the regulation of the renal excretion of water. Starling and Verney(20) and Verney(22), as a result of their isolated heart-lung-kidney experiments, suggest that the hormone of the posterior lobe might quite well be involved in the regulation of the renal water output, and that diabetes insipidus is the result of some pathological disturbance of this mechanism.

Recently the author(11) has described a method of obtaining, in an experimental preparation, water diuresis which is identical in every respect with that resulting from the ingestion of water in the normal intact animal. The experiments described below were carried out in order to determine to what extent complete extirpation of the pituitary body would influence renal secretion after the possibility of irritative factors in the region of the third ventricle was removed; and to reconcile, if possible, the findings of Starling and his co-workers with those of the opposite school.

Experimental technique.

All the experiments have been performed on hypophysectomized and decerebrate dogs. In the majority of cases the animals were given no food or water for the 12 hours preceding the experiment. Morphia and non-volatile anæsthetics were not used; the operative manipulations being performed under chloroform-ether anæsthesia which was removed as soon as was feasible. After ligature of the carotid arteries and the insertion of a tracheal cannula the pituitary body was removed by the double decompression method of Paulesco(17). Usually it was possible to remove the whole of the gland in a single piece. The animal was then decerebrated, the cut being made well posterior to the tuber cinereum, and all of the brain substance anterior to the plane of transection removed from the skull. This procedure served the twofold purpose of rendering unnecessary the use of anæsthetics and of eliminating the possibility of irritative substances, generated by a tuber cinereum injured during hypophysectomy, finding their way into the general circulation. The pituitary body was removed before decerebration to avoid the possibility of small particles of the gland (which might influence the nature of the experiment) remaining attached to the floor of the pituitary fossa. The completeness of the operation was always checked by a post-mortem examination. At least an hour was allowed for the animal to recover from the anæsthesia and the shock of decerebration before experimental records were taken. Urine was collected

from extraperitoneal ureteral cannulæ. Chlorides were estimated by Hardy's modification of the Volhard method.

Experimental results.

A. Urine excretion after hypophysectomy and decerebration.

The results of 12 successful experiments are available and of two in which excessive hæmorrhage, as a result of decerebration, caused a very low blood-pressure. The rate of urine excretion was followed for periods varying between two and six hours before further experimental manipulations were made. The average urine flow of these experiments was 1·86 c.c. per ten minutes, the highest being 6·7 c.c. and the lowest being 0·5 c.c. per ten minutes. The animals which showed a higher rate of urine output than the average were those which had received water a few hours previous to the experiment. In these the rate of urine excretion usually fell within a few hours. In no case was a marked increase in urine output encountered after the animal had recovered from the shock of decerebration. The rate of urine flow of the normal intact animal under similar conditions is between 1 and 4 c.c. per ten minutes; hence it may be concluded that complete removal of the pituitary body followed by decerebration has no effect upon the secretion of urine during the first eight hours after the operation. Furthermore, since removal of the pituitary body, in both chronic and acute experiments, is by no means always followed by polyuria it would appear that the absence of the posterior lobe of the pituitary is not, in itself, responsible for diabetes insipidus. The chloride content of the urine of four experiments averaged 0·109 g. p.c. (as NaCl), while a bladder sample collected from one animal contained 0·117 g. p.c. The blood-pressure, heart rate and respiration were satisfactory in the majority of these experiments, although frequently Cheyne-Stokes respiration was encountered. Most of these preparations were used later for the experiments described in the following sections.

B. Water diuresis after hypophysectomy and decerebration.

In nine of the animals just described water was later slowly injected into the duodenum or small intestine. Providing that the blood-pressure was satisfactory the urine flow began to rise within 20 to 30 minutes, reaching a maximum during the second hour and remaining at a high level until a greater portion of the excess water absorbed from the gut had been excreted. A typical experiment is given in Table I. During

the hour before giving the water 25·0 c.c. of urine were excreted. The abdomen was then opened and 350 c.c. of warm water passed into the duodenum over a period of 40 minutes. The operation caused an initial lowering of the urine flow so that at the end of the hour, although the rate of secretion had risen to over twice the previous flow, the total amount of urine excreted was only 15·5 c.c. During the next three hours 106·0, 111·5 and 52·0 c.c. of urine were excreted. Deducting 75·0 c.c. as the basal flow during this period 212·5 c.c. of the 350·0 c.c. placed in the duodenum were eliminated. Post-mortem examination of the animal indicated that the pituitary body and tuber cinereum had been completely removed.

A true flood diuresis only failed to occur in the two experiments already referred to in which excessive hæmorrhage had reduced the blood-pressure, but even in these the rate of excretion rose from 1·4 to 8·0 c.c. and from 0·5 to 6·0 c.c. per ten minutes. When due allowance is made for the hæmorrhage attendant upon operations in the dog in which the circle of Willis is ruptured, these experiments justify the

TABLE I. Water diuresis after hypophysectomy and decerebration. Operation completed at 11.30.

Time	Rate of urine flow c.c./10 min.	Remarks
12.10–12.30	4·45	
12.30–12.50	3·95	
12.50–13.10	4·60	
13.10–13.40	1·50	13.10 350 c.c. warm water into duodenum
13.40–14.00	2·40	
14.00–14.10	6·00	
14.10–14.30	11·50	
14.30–14.50	19·30	
14.50–15.10	22·30	
15.10–15.40	22·00	
15.40–16.00	17·70	
16.00–16.10	10·00	
16.10–16.30	15·00	
16.30–16.50	7·00	
16.50–17.00	4·00	
17.00–17.10	2·00	

Animal killed at 17.10. Heart rate, blood-pressure and respiration good throughout experiment.

statement that the hypophysectomized and decerebrate dog is capable of eliminating quantitatively any excess water absorbed from the intestinal canal. During the height of the diuresis the urinary chlorides fell to a very low level (less than 0·03 g. p.c.). The blood-pressure was recorded in several experiments and was found to remain practically constant. In one case the initial pressure was 100 mm. Hg, but fell to under 90 mm. during the height of the diuresis. No correlation could be

obtained between the height of diuresis and the concentration of hæmoglobin, a fact which has frequently been noticed during water diuresis in man and the intact animal. Pituitrin has an antidiuretic action as in the normal animal.

C. Diuresis from intravenous hypotonic saline after hypophysectomy and decerebration.

Thompson(21) and Magnus(15) obtained a well-marked diuresis after the intravenous injection of 0·6 p.c. saline, in some cases more excess urine being excreted than the amount of saline given. On the other hand Haake and Spiro(13), Starling and Verney(20), Fee(10) and many others state that isotonic and hypotonic saline, when given in moderate amounts, do not increase the urine flow in proportion to the amounts given. It is of interest to note that both Thompson and Magnus worked with whole animals which had been lightly anæsthetized with morphia some hours previous to the experiments and that choloroform-ether anæsthesia was only used when absolutely necessary. Haake and Spiro and other workers have used urethane or some other non-volatile anæsthetic.

On giving 300 c.c. of 0·6 p.c. saline solution intravenously, at a rate of 5 p.c. per minute, to a hypophysectomized and decerebrate dog the urine flow rose immediately from 1·0 c.c. to a maximum of 34·0 c.c. per ten minutes an hour after the saline infusion was begun. This diuresis was maintained until 230 c.c. of the excess fluid had been excreted. On giving 0·3 p.c. saline under the same conditions no immediate increase in urine flow was apparent, but an hour later the flow increased approximately five times and was maintained at this level until the blood-pressure of the animal began to fail. In an anæsthetized (chloralose) animal only 48 c.c. of excess urine were excreted during the next three hours after 300 c.c. of 0·6 p.c. saline were given intravenously at a rate of 5 c.c. per minute. These experiments indicate that the divergent results obtained after the intravenous injection of hypotonic saline depend solely upon the type of preparation used. The chlorides during such a diuresis do not fall to the same extent as during a water diuresis. Markedly hypotonic solutions are probably, as suggested by Cushny(7), immediately absorbed by the tissues from which they slowly escape into the blood and are excreted. After complete hypophysectomy the kidney is, therefore, capable of reacting to the changes in the composition of the blood attendant upon the injection of hypotonic saline.

D. The effect of denervation after hypophysectomy and decerebration.

The effect of denervating one kidney in the hypophysectomized and decerebrate dog is shown in Table II. In this experiment the nerve

TABLE II. Urine excretion after denervating kidney in hypophysectomized and decerebrate dog.

11·75 kilos, ♂. Pituitary body removed and decerebrated 14.40. Left kidney denervated 15.00. Extraperitoneal ureteral cannulæ inserted 15.30.

Left kidney (denervated)			Right kidney (control)	
Urine c.c./10 min.	NaCl g. p.c.	Time	Urine c.c./10 min.	NaCl g. p.c.
4·1	—	15.30–15.40	0·5	—
6·0	0·029	15.40–15.50	0·5	0·105
5·2	0·029	15.50–16.00	1·0	0·073
5·5	0·035	16.00–16.10	1·3	0·073
		Five units Parke Davis pituitrin injected.		
1·9	—	16.10–16.20	1·1	—
2·5	0·053	16.20–16.30	1·1	0·117

supply to the left kidney was destroyed by carefully removing the outer coat of the renal artery throughout its whole length. As a result of this procedure the urine flow from the denervated kidney was over six times that of the right kidney, the nerves of which were left undisturbed. The chloride content of the urine secreted by the denervated kidney was also much lower than that of the control. These results confirm those of Marshall and Kolls(16). Five units of pituitrin (Parke Davis) were then injected, and the diuresis resulting from denervation was reduced until the urine flow was practically the same as that of the normal kidney, while the output of the normal kidney remained unaltered. Concomitant with the reduction in urine flow there was an increase in the percentage output of urinary chlorides. As the effect of the pituitrin wore off the flow of the denervated kidney once more became larger than that of the control.

This experiment indicates that pituitrin, by its action on the kidney, may reduce a diuresis which has been initiated by changes in the renal circulation resulting from denervation, although the same amount has no appreciable effect upon the volume of urine excreted by a normal kidney. It is well known that in the human subject, if the urine flow is much reduced, the injection of pituitrin will not markedly alter the rate of flow although the chlorides may be influenced to some extent.

Discussion.

While these experiments do not demonstrate the cause of diabetes insipidus they indicate that the removal of the posterior lobe of the pituitary body is not necessarily attended by polyuria. Moreover, it is probable that this disorder is not a pathological disturbance of the mechanism which is normally responsible for the regulation of the renal elimination of excess water. The kidney is able to adjust its rate of excretion to suit the requirements of the body after all possibility of the intervention of the pituitary body or base of the brain has been removed by complete hypophysectomy followed by decerebration. Such animals react to ingested water and to the blood changes initiated by administering intravenous hypotonic saline, and after these changes have been compensated for by the elimination of excess urine, the flow will once more return to the basal level. As has been previously indicated, this adaptation to changes in the water content of the tissues and blood is not manifested after non-volatile anæsthetics and morphia.

The behaviour of the denervated kidney in such a preparation is of exceptional interest as it offers an alternative explanation for the behaviour of the isolated kidney perfused by the heart-lung preparation. The essential differences in the environment of an isolated kidney and of a denervated kidney in a hypophysectomized and decerebrate preparation are that in the former the kidney has been removed from whatever influence the majority of tissues may exert on the blood; and that defibrinated blood, rather than normal blood, is used as a perfusion medium. That defibrinated blood is a powerful vasoconstrictor is well known, and it has been shown (9) that whatever is responsible for these vasoconstrictor properties is removed by circulating the blood through a heart-lung preparation. If a kidney is inserted into a heart-lung preparation immediately after the latter has been made little or no urine is produced for some minutes, presumably until the vasotonins of the freshly defibrinated blood have been destroyed. If, on the other hand, the blood is allowed to circulate for some time before the kidney is shunted into the circuit the urine flow starts immediately; large amounts of a very hypotonic urine being excreted. This abnormal urine flow is checked by adding pituitrin to the circulating blood. Pituitrin, however, will also check the abnormally high urine flow of a denervated kidney in the hypophysectomized and decerebrate dog in exactly the same manner. It is suggested, therefore, that the behaviour of the isolated kidney, perfused by the heart-lung preparation, is due solely to complete denervation consequent upon its removal from the animal.

Summary.

1. The effect of total hypophysectomy upon the renal excretion of chlorides and water was investigated in decerebrate dogs. The use of morphia and non-volatile anæsthetics was avoided.

2. Total hypophysectomy, followed by decerebration, does not result in either an increased flow of urine or in a marked change in the output of chlorides.

3. The kidneys of such a preparation react to excess water absorbed from the alimentary canal in the same manner as do those of the normal intact animal. Such a diuresis is inhibited by pituitrin.

4. After total hypophysectomy intravenous 0·6 p.c. saline is excreted quantitatively by the kidneys during the next few hours.

5. Denervation of the kidney, after hypophysectomy and decerebration, results in the production of large amounts of markedly hypotonic urine similar to that excreted by the isolated kidney, perfused by the heart-lung preparation. Pituitrin reduces this increased output to normal and raises the percentage amount of chlorides excreted.

6. It is suggested that complete denervation, resulting from isolation of the kidney, may account for the formation of large amounts of hypotonic urine by the heart-lung-kidney preparation.

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THE REPLACEMENT OF DEPLETED ADRENALINE IN THE SUPRARENALS.

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The physiological control of the discharge of adrenaline from the suprarenal glands of cats was shown by Elliott(1) to depend on the integrity of the splanchnic nerves. The question whether or not the production of adrenaline in the glands also depends on nervous influences was left undecided, although Elliott tentatively observed that "anabolism seems to proceed without central control" in view of the fact that, following the section of the splanchnic nerves on one side, the denervated gland was invariably found to contain as much, if not more, adrenaline than its fellow.

The following research has been carried out in order to determine if nervous control is essential for the production of adrenaline.

It was necessary to possess some controllable means of producing, in the first place, a definite degree of exhaustion of the store of adrenaline in the glands from which the subsequent production during recovery could be assessed. Attempts were made to utilize the methods shown by Elliott to produce exhaustion of the glands, namely, the fright caused by morphia or β-tetra hydronaphthylamine hydrochloride, but these methods had to be discarded owing to the variability in the reaction of different animals to these drugs(2). It should be stated, however, that in several recovery experiments following exhaustion by these methods indications were obtained that adrenaline production can take place in the denervated glands. Finally the influence of lowered body temperature was tried and found satisfactory.

Several observers have placed on record physiological(3, 4) and histological(5) evidence for the discharge of adrenaline from the glands when the animal is subjected to cold. Preliminary experiments showed that provided the application of external cold is followed by a marked drop in the body temperature of the animal, then in the normally

innervated gland the adrenaline store is depleted(6). Cold was therefore adopted as a means of producing exhaustion of the glands.

Experimental method. The problem has been approached by the direct method of determining the relative total adrenaline content of the glands under controlled conditions for producing exhaustion and for permitting recovery in innervated and denervated glands.

Since Elliott(1) proved that the adrenaline content of the two glands remains equal after section of the splanchnic nerves on one side, provided the animal is quiet and undisturbed, it is possible to assess the degree of exhaustion, or of recovery following exhaustion, if the relative quantities of adrenaline can be estimated accurately. The colorimetric method of Folin, Cannon and Denis(7) was employed for this estimation. Preliminary tests showed that very great care must be taken to ensure that in every detail of time, temperature and manipulation, the glands to be compared are treated in the same manner. The work of Baker and Marrian(8) showed that the rate at which the colour develops, after the "uric acid reagent" and saturated solution of sodium carbonate have been added to the extract of the gland, depends on the temperature, and that for a temperature of 20° C. the maximum colour develops in 20 minutes.

Estimation of the adrenaline content of glands (0·1–0·3 *g. weight*). At the termination of the experimental period the animal was killed quickly by chloroform and coal gas. The glands were immediately removed, weighed and the adrenaline estimated simultaneously in each gland according to the following directions:

1. Place glands with equal quantities of silver sand and 1 c.c. *N*/10 HCl in two 3-inch evaporating dishes, grind thoroughly for three minutes, then add 3 c.c. *N*/10 HCl, grind a few seconds, wash pestle with 5 c.c. distilled water and transfer with further 5 c.c. of water to a small Erlenmeyer flask.

2. Boil 3 minutes over equal gas flames, then add 1 c.c. 10 p.c. sodium acetate and boil ½ minute; cool quickly under tap.

3. Filter through Büchner, using 7 c.c. H_2O for washing. Transfer filtrate to 50 c.c. graduated flask, wash in with 3 c.c. H_2O.

4. Add 2 c.c. "uric acid reagent," 10 c.c. saturated sodium carbonate and make up to 50 c.c. with water.

5. Place in water bath at 20° C. for 20 minutes and then compare in Dubosc colorimeter for the relative total amounts of adrenaline in the glands.

For determination of the actual amounts of adrenaline, a parallel

colorimetric estimation was simultaneously carried out with a standard solution of uric acid.

The accuracy of this method was checked with a standard solution of pure adrenaline hydrochloride (British Drug Houses, Ltd.). The error due to the presence of blood was found to be negligible.

Exhaustion of adrenaline store by cold. Experiments were carried out to ascertain the conditions under which cold depletes the adrenaline store in the glands and to provide data for a curve of progressive exhaustion.

The animals (male cats) were kept in a warm animal house, and after each animal had settled down to cage life operative section of the splanchnic nerves and the nerves to the semilunar ganglion on the left side was performed. The animals were then allowed to recover undisturbed for a few days at a room temperature of 65° F. in different cages separated by sacking and on a uniform diet of meat and milk. Under these conditions at the end of a day or two the denervated and innervated glands were almost equally loaded with adrenaline. This fact, already observed by Elliott(1), was confirmed by the experiments shown in Table I.

TABLE I. Relative content of adrenaline in suprarenal glands.

Condition	Relative content of glands	
	Right	Left
Normal animal	100	108
"	100	100
"	100	104
"	100	97
Left splanchnics cut 12 days	100	108
Left splanchnics cut, animal killed after operation	100	98·5
Left splanchnics cut by ligature	100	97·5

This and subsequent tables do not give the absolute amounts of adrenaline present in the right and left glands, but show their relative contents in terms of the content of the gland on the side taken as normal which is expressed as 100 p.c. There is a slight variation in the relative equality of content of the glands, but for the purposes of this investigation it is sufficiently accurate to assume that the glands start with an equal content of adrenaline.

On the day of a cold experiment the animal was not fed. After taking its deep rectal temperature it was transferred to a room at 0° C. The fur and skin up to the neck were wetted and the animal was then left undisturbed except when its rectal temperature was taken. After the required interval the animal was killed quickly and the adrenaline

content of the glands compared. Table II gives the results of this series of experiments.

TABLE II. Exhaustion of suprarenal glands by cold.

Exp.	Duration of external cold hr.	Duration of external cold min.	Total fall in temperature (deep rectal) °C.	Degree hr. internal cold	Average intensity of internal cold. Degree hr. per hr.	Relative content of glands: Denervated	Relative content of glands: Innervated
1	4	35	5·3	19	4·1	100	71·0
2	4	15	4·5	14	3·29	100	65·5
3	1	30	12·0	9·5	6·3	100	85·5
4	6	10	5·0	13	2·1	100	82·0
5	5	40	6·0	12·5	2·2	100	62·5
6	8	10	0·5	0·5	Nil	100	101·0
7	7	30	1·4	2·0	0·26	100	115·0

As regards "degree hours internal cold," a degree hour (1° hr.) may be defined as a difference in temperature from the initial normal temperature of 1° C. persisting for a period of 1 hour. If the rectal temperature of an animal were found to remain 2° C. below its normal temperature for a period of 1 hour the cold stimulus to which it had been subjected would be, according to this definition, 2° hours internal cold.

A continuous record of body temperature was not made, but readings of the deep rectal temperature were taken at intervals of approximately 1 hour while the animals were exposed to cold. From the data thus obtained the temperature charts shown in Figs. 1 and 2 were constructed. The area between the temperature curve and the normal temperature base line in Figs. 1 and 2 gives the number of degree hours internal cold. The intensity of the cold stimulus is given in terms of degree hours per hour in Table II. It will be observed that, although the number of hours exposure to external cold may be considerable, unless there is a resulting adequate cooling there is not a depletion of the adrenaline store of the innervated gland as compared with the denervated gland, the content of which is taken as 100.

In animal 6 which had been fed in the morning in error, shivering caused the body temperature to rise slightly and apparently give the total chilling a small negative value in spite of the ultimate fall in temperature of 0·5° C. In animal 7 the temperature curve is also irregular, the final result being a slight fall representing 2° hours internal cold at an intensity of 0·26° hours per hour.

In the normal animal there must be a balance which is converted to a depletion by conditions which cause an excess of discharge of adrenaline over production. On the other hand, it is possible that a weak stimulus,

of a kind which if intensified would lead to such depletion of adrenaline, may in itself produce a preliminary production equal to or in excess of

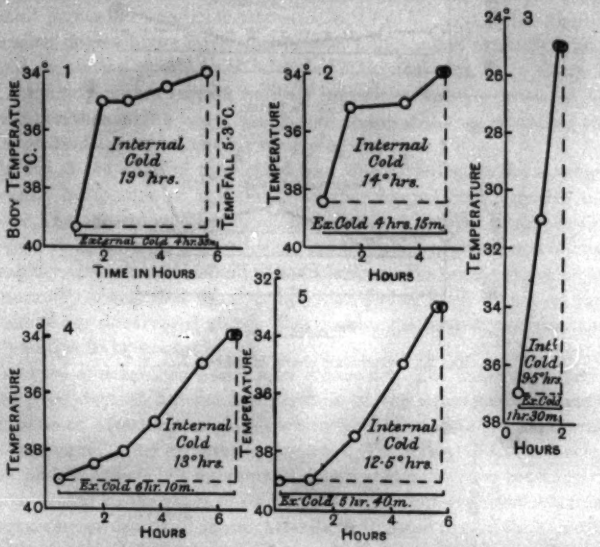

Fig. 1. Temperature charts of animals 1, 2, 3, 4, 5, during exhaustion of suprarenal glands by cold. In all cases male cats were used.

A degree hour, 1° hr., = a difference in temperature of 1° C. from normal persisting for a period of 1 hour. The degree of internal cold is expressed in these units. Temperature = deep rectal temperature.

discharge and so account for the fact that in experiments Nos. 6 and 7 there was no depletion of the adrenaline store of the innervated glands. Cramer's observations (5) of the histological changes in the suprarenals of the mouse when subjected to cold support this view.

On plotting the degree of exhaustion of the innervated gland against the duration of the external cold stimulus, no curve of progressive exhaustion is obtained owing to the fact that animals 6 and 7 showed no depletion in spite of considerable external cold. A similar result is obtained when the degree of exhaustion is plotted against the total fall in body temperature. The rapid drop in temperature of 12° C. in 1½ hours in the case of animal 3 did not result in a proportionate degree of

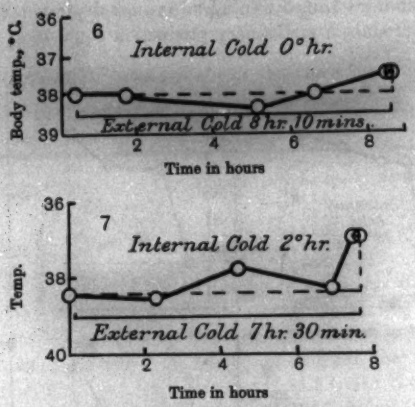

Fig. 2. Temperature charts of animals Nos. 6, 7.

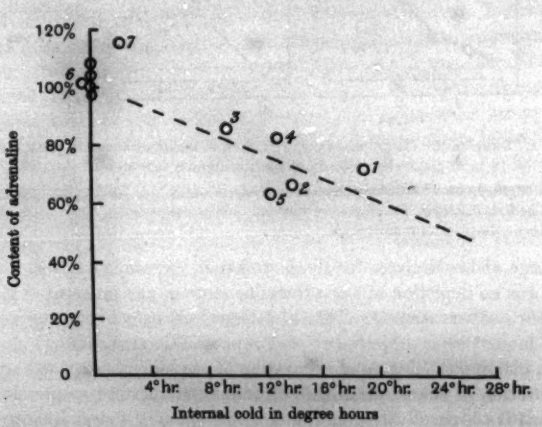

Fig. 3. Chart showing progressive exhaustion of adrenaline store of innervated glands by internal cold. The numbered points indicate the content of the innervated glands of animals 1–7 expressed as a percentage of the content of the denervated glands. The four unnumbered points indicate the relative content of the glands of control animals which were not subjected to cold.

exhaustion of the adrenaline store. When, however, the degree of exhaustion is plotted against the number of degree hours internal cold with which the bodies of the animals had attempted to cope, these aberrant points fall approximately into line (Fig. 3), because the figures expressing degree hours internal cold take into account the actual loss of heat regulation reached. It is evident therefore that it is internal cold, probably within certain limiting intensities, *i.e.* the lowering of the temperature of the body, which acts as the stimulus producing an excess of discharge of adrenaline over production.

In Fig. 3 the slope of the dotted line roughly indicates the average increasing exhaustion of the glands as the internal cold stimulus increases. The general result is clear, namely, that with an internal cold stimulus of 12 or more degree hours, the body temperature not falling too rapidly or too low, there will be a depletion of the adrenaline store of the normally innervated gland representing some 20 or 30 p.c. of the content of the denervated gland. This enables the recovery production of adrenaline to be assessed.

Recovery of innervated glands after exhaustion. The following experiments were devised for the investigation of the subsequent recovery production of adrenaline in glands which were allowed to remain normally innervated after exhaustion by cold. The experimental animals, which had had their left suprarenals denervated some days previously, were subjected to the effect of cold such as had produced exhaustion in the experiments described above. After being exposed to cold the animals were returned to the warm animal house, rubbed down with warm towels, placed near a hot water bottle and given warm milk to drink. Under these conditions they rapidly regained their normal temperature and

TABLE III. Recovery of innervated glands after exhaustion.

Experiment No.	8	9	10
Duration of external cold	6 hr. 55 min.	7 hr. 10 min.	6 hr.
Fall in body temperature (deep rectal)	12·1° C.	4·6° C.	3·8° C.
Degree hours internal cold	33·5	13·5	13
Average intensity of internal cold. Deg. hr. per hr.	4·8	1·88	2·16
Estimated relative content of glands after exhaustion:			
Denervated	100	100	100
Innervated	32	73	74
Innervated recovery period	18 hr. 25 min.	69 hr.	71 hr.
Relative content of glands after recovery:			
Denervated	100	100	100
Innervated	79	109·5	97·5
Note on recovery	Incomplete	Complete	Almost complete

behaviour. Then after varying periods of time the animals were killed quickly and the relative content of adrenaline in their glands estimated.

Table III gives the results of the innervated recovery experiments.

It will be observed that 18 hours under the conditions of the experiment No. 8 were insufficient for the innervated gland to replenish its normal store of adrenaline. In the subsequent experiments the recovery period was increased to approximately 3 days in order to give ample opportunity for full recovery. It is probable that animal 9 had replenished its adrenaline store before 69 hours had elapsed.

Recovery of denervated glands after exhaustion. In the case of the denervated recovery experiments animals in which the left glands had been denervated some days previously were exposed to cold, then quickly put under an anæsthetic and the nerves to the right gland cut. They were then returned to the warm animal house and treated as in the case of the innervated recovery experiments. A period of recovery of approximately 3 days was allowed, after which the routine estimation of the relative content of adrenaline in the glands was carried out. The data for these experiments are given in Table IV.

TABLE IV. Recovery of denervated glands after exhaustion.

Experiment No.	11	12	13
Duration of external cold	5 hr. 35 min.	7 hr. 10 min.	6 hr.
Fall in body temperature (deep rectal)	4·5° C.	7·8° C.	9·8° C.
Degree hours internal cold	12·5	23	21·5
Average intensity of internal cold. Deg. hr. per hr.	2·24	3·48	3·58
Estimated relative content of glands after exhaustion:			
Denervated left	100	100	100
Innervated right	75	54	58
Denervated recovery period	71 hr.	68 hr. 30 min.	69 hr. 15 min.
Relative content of glands after recovery:			
Denervated left	100	100	100
Denervated right	100	108·5	93
Note on recovery	Complete	Complete	Almost complete

Experiment No. 9 in Table III and 12 in Table IV and similarly 10 and 13 are two pairs of parallel experiments in which the animals in each pair were subjected to identical cold conditions at the same time. There is considerable variation in the degree of resistance offered by different animals to the influence of external cold, and therefore in order to make the test as conclusive as possible the animals selected for the denervated recovery experiments (Nos. 12 and 13) were the ones which had developed the greatest degree of chilling. The temperature charts of these four

animals and the graphical representation of the exhaustion and recovery of their glands are shown in Figs. 4 and 5. On comparing the figures

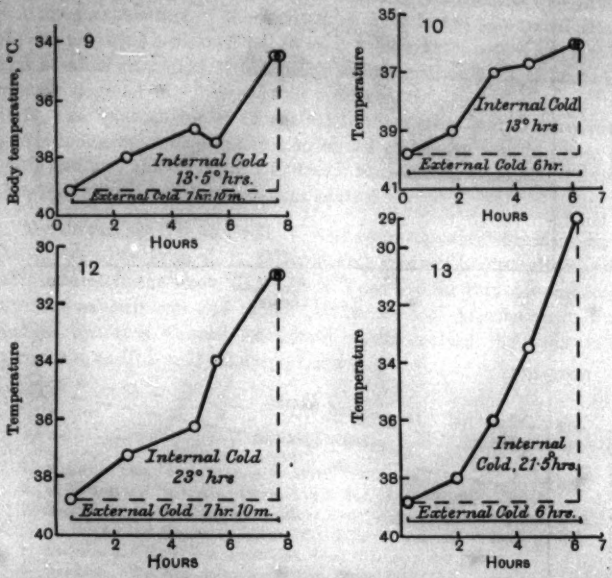

Fig. 4. Temperature charts of animals Nos. 9, 10, 12, 13, during exhaustion of innervated glands by cold. In Nos. 9 and 10 the innervated glands were allowed to recover after exhaustion. In Nos. 12 and 13 the innervated glands were denervated after exhaustion and then allowed to recover. Exp. Nos. 9 and 12, and similarly Nos. 10 and 13, were carried out under identical conditions.

for the relative content of adrenaline in the glands after innervated and denervated recovery in these four experiments, it is evident that there is no significant difference in the final results, namely, that in each case replacement of the depleted adrenaline store of the glands has occurred.

It is therefore held that these experiments prove that the production of adrenaline can take place after denervation of the suprarenal glands.

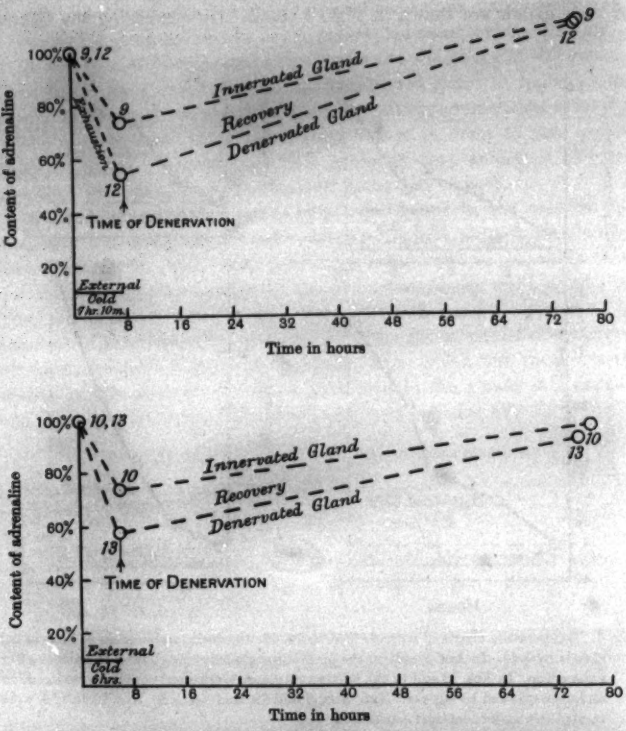

Fig. 5. Chart showing exhaustion of adrenaline store of innervated glands of animals 9, 10, 12, 13, and subsequent recovery production of adrenaline.

No. 9. Exhaustion by 13·5° hr. internal cold in 7 hr. 10 min. followed by recovery for 69 hr.

No. 12. Exhaustion by 23° hr. internal cold in 7 hr. 10 min. followed by denervation and then recovery for 68 hr. 30 min.

No. 10. Exhaustion by 13° hr. internal cold in 6 hr. followed by recovery for 71 hr.

No. 13. Exhaustion by 21·5° hr. internal cold in 6 hr. followed by denervation and then recovery for 69 hr. 15 min.

SUMMARY.

1. The application of external cold is a controllable method of producing exhaustion of the adrenaline store of the suprarenal glands of cats, provided a marked fall in body temperature occurs and persists over a sufficient period of time. The effective stimulus is estimated in terms of degree hours internal cold produced.

2. The replacement of adrenaline in innervated and in denervated glands following partial exhaustion of the glands by cold has been investigated and the conclusion is drawn that the replacement of adrenaline may proceed without nervous control.

My thanks are due to Dr G. V. Anrep for drawing my attention to this problem, to Prof. T. R. Elliott and Prof. C. Lovatt Evans for their interest in the work, to Dr M. G. Pearson for valuable assistance with the experiments and to the British Medical Association and the Medical Research Council for grants which enabled the work to be carried out at University College, London.

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THE CORONARY CIRCULATION.

III. The dependence of changes in the coronary blood flow on cardiac and local vascular factors.

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The study of the blood supply to the heart muscle presents certain difficulties which are not encountered in similar investigations on most organs. Plethysmographic methods, which are helpful in investigations upon the blood supply to many organs, are useless in the case of the coronary circulation. In experiments on the whole animal in which the coronary blood flow is measured by collecting the blood which emerges from a cut cardiac vein, or from the coronary sinus, the results are always open to criticism because of the great difficulty of controlling the arterial blood-pressure and the output of the heart. On account of the extreme dependence of the coronary blood flow on the aortic blood-pressure, it is necessary (probably more necessary here than in the case of any other organ) to use various devices which permit of a perfect control of all the mechanical factors of the circulation. It is therefore not surprising that most physiological and pharmacological studies of the coronary circulation have been made on the isolated perfused heart, or on the heart-lung preparation. The object of this communication is to show that even the degree of control which is obtainable in these preparations is in many cases insufficient, and that an augmentation or diminution of the coronary blood flow under the influence of some physical or chemical agents does not necessarily indicate, as in the case of most organs, a respective vaso-dilator or vaso-constrictor reaction. In other words, records of the outflow per minute from a coronary vein, or of the inflow per minute into a coronary artery, are often misleading without further analysis, even under conditions of a uniform blood-pressure. The principle of the necessary analysis has been described before (1, 2); it is based on the application of the hot-wire anemometer. It has been shown in previous experiments (3, 4) that the circulation in a perfused coronary artery depends largely on the heart beat, that when the strength of the contraction is increased by any means whatever the diminution of the

coronary blood flow during each systole becomes more conspicuous, and that in extreme cases there may be a regurgitation of blood into the arterial system. Therefore, at a constant coronary pressure, the blood flow per minute is smaller the stronger the contraction of the heart. The effect of changes of heart rate on the coronary blood flow is determined by the relative duration and by the strength of systole.

Various physical and chemical agents which affect the coronary blood vessels usually also affect the heart muscle and, since the experimenter has no means of controlling the strength of the heart beat, he must at least have some method of measuring it, so as to be able to analyse the mechanism by which any change in the coronary blood flow is produced—whether it is due to the action of the agent on the heart muscle or on the coronary blood vessels or both, and in the latter case to what extent each of the two factors is responsible. In order to demonstrate the dependence of the changes in the coronary blood flow on the cardiac and local vascular factors, a comparison was made between the action of four substances, two of which have a predominantly vascular effect, while the other two are known to have a considerable effect on the heart muscle, and also some effect on the coronary blood vessels. The first two substances were (*a*) amyl nitrite, which conspicuously increases the coronary blood flow, and (*b*) pituitary extract, which diminishes the coronary blood flow; in small doses these drugs are not known to have any direct effect on the heart muscle. The other two substances were (*c*) adrenaline, which considerably strengthens the heart beat, and (*d*) carbon dioxide, which weakens it. As regards their action on the coronary blood flow, opinions are divided about the action of adrenaline, but carbon dioxide is generally considered to increase the coronary blood flow.

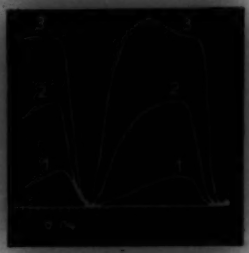

Fig. 1. Superimposed corrected hot-wire records of the inflow of blood into the left coronary artery. Heart-lung preparation. All the mechanical conditions of the circulation and the heart rate were maintained constant. The mean aortic blood-pressure and the perfusion pressure were kept at 100 mm. Hg.

1. After injection of 0·1 c.c. of B.D.H. pituitary extract into the blood perfusing the coronary artery.
2. Normal record.
3. After injection of 1 c.c. of saline solution saturated with amyl nitrite.

The respective coronary blood flows were 0·09 c.c., 0·37 c.c. and 0·85 c.c. per cardiac cycle. The heart rate in this and all the following tracings was controlled by rhythmic stimulation of the right auricle.

An anaylsis of the effect of amyl nitrite and of pituitary extract is

shown in Fig. 1, in which a normal hot-wire record is also given for comparison. It can be seen that in the three records there is a complete arrest of the coronary blood flow during the period of systole. During the period of diastole the blood flow is increased by amyl nitrite and decreased by pituitary extract.

Fig. 2 is an original record taken before and after administration of pituitary extract.

(a) (b)

Fig. 2. Original hot-wire records and optical registration of the aortic blood-pressure: (a) normal record; (b) 15th and 16th heart beat after injection of 0·1 c.c. of pituitary extract into the perfused left coronary artery. The coronary blood flows per cycle were 0·66 c.c. and 0·32 c.c. respectively. Time in this and all the following tracings 0·04 sec.

The shift of the level of the diastolic part of the record may be taken to represent the most accurate measure at present possible of the changes in the resistance of the coronary system, *i.e.* vaso-dilation or vaso-constriction. The rate at which the coronary blood vessels are refilled, after having been emptied by the preceding contraction of the heart, is greater when they are dilated. In the case of considerable dilation, the refilling is so rapid that the maximum rate of inflow of blood takes place in the first part of diastole, and the true diastolic level of the record is only reached later. In the case of small doses of amyl nitrite and of pituitary extract the changes in the coronary blood flow, as recorded by measuring the inflow of blood per minute, are proportional to the shift of the diastolic part of the hot-wire record. In some experiments amyl nitrite in large doses had a strengthening effect on the heart itself, as can be seen from Fig. 3. In this case the cardiac contraction was not

sufficiently strong to stop the inflow of blood into the perfused coronary artery. On administration of amyl nitrite the diastolic level of the

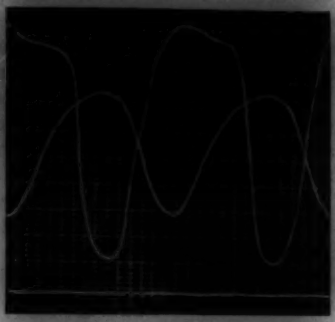

Fig. 3. Original hot-wire records: (*a*) normal; (*b*) after injection of 1 c.c. of saline saturated with amyl nitrite. The coronary perfusion pressure was 100 mm. and the mean aortic blood-pressure 75 mm. Hg. Both records were taken on the same film, the time-marker being removed during the second exposure.

coronary inflow was shifted in the upward direction, and at the same time the diminution of the blood flow during systole became more pronounced. These changes in the hot-wire record indicate the occurrence of the usual (with amyl nitrite) dilation of the blood vessels, and also a more forcible compression of the blood vessels during systole. It is difficult to decide whether this strengthening of the contraction is due to a direct effect of amyl nitrite on the heart muscle itself, or indirectly to a more vigorous coronary circulation following the administration of the drug. This effect was observed in a few cases only.

The above experiments show that the main effect of these two drugs is to shift the level of the diastolic part of the hot-wire record, which conforms with the predominantly vascular nature of their action. Conditions are however not often as simple as these. When the agent used has a greater effect on the heart muscle, it becomes increasingly difficult to determine its effect on the coronary blood vessels. I believe that a great number of controversial statements concerning the action of such potent cardiac drugs as adrenaline and strophanthin are due to these difficulties. Almost within a month, strophanthin has been claimed to be a coronary vaso-constrictor and a coronary vaso-dilator substance. As regards adrenaline, some observers deny that it has any effect on

the coronary circulation, others ascribe to it purely vaso-constrictor properties, others regard it as a coronary vaso-constrictor substance in small doses and a vaso-dilator substance in large doses, and still others ascribe to it vaso-dilator properties only(6). These conflicting results are due to the fact that the blood flow per minute through the coronary system has been taken as a measure of coronary vaso-dilation or of vaso-constriction. In the action of adrenaline there are at least three factors which have to be considered. The net result on the coronary blood flow is the algebraic summation of the effects of the change in heart rate, the change in strength of contraction, and the vascular changes proper. The first factor can be eliminated because the heart rate can be controlled by the experimenter. The extent of the effect on the coronary circulation of the change in the strength of the beat and of local vascular phenomena must however be determined by analysis on the same lines as that which was applied in the case of amyl nitrite and pituitary extract.

The following is an example of such an analysis of the effect of adrenaline on the inflow of blood into the perfused left coronary artery in the heart-lung preparation. The coronary artery was perfused with a pressure at which the contraction of the heart was able to stop the coronary circulation during each systole. The hot-wire record of this period is shown in Fig. 4 (*a*). Adrenaline was then injected into the

(*a*) (*b*)

Fig. 4. Original hot-wire records: (*a*) before, (*b*) after injection of 0·4 c.c. of 1 : 50,000 solution of adrenaline chloride into the left coronary artery. The mean aortic and the perfusion pressures were 100 mm. Hg. Coronary blood flow per cycle 0·52 c.c. and 0·72 c.c. in (*a*) and (*b*) respectively.

perfused artery; the corresponding hot-wire record in Fig. 4 (*b*) shows that the effect is somewhat like that produced by amyl nitrite but smaller.

The observation was repeated under conditions in which the cardiac contraction was not strong enough to stop the inflow into the coronary artery. This weakening of the contraction was obtained by lowering the aortic blood-pressure to about 30 mm. of mercury below the perfusion pressure. After the injection the coronary blood flow at first diminished. The hot-wire record of Fig. 5 shows that this diminution

(*a*) (*b*) (*c*)

Fig. 5. (*a*) Normal record. (*b*) The 7th end, (*c*) the 30th heart beat after injection of 0·4 c.c. of a 1 : 50,000 solution of adrenaline. Mean aortic blood-pressure 70 mm., perfusion pressure 100 mm. Hg. Coronary blood flows per cycle 0·85 c.c., 0·60 c.c. and 0·98 c.c. in (*a*), (*b*) and (*c*) respectively. The optical manometer recording the aortic blood-pressure was screened off in (*a*) and (*b*).

is due to a more pronounced restriction of the coronary circulation by the strengthened contraction of the heart. At this stage the diastolic level of the inflow of blood is not much affected. But later vaso-dilation takes place as can be seen by the upward shift of the diastolic portion of the record. These observations make it evident that, although the dilation of the coronary blood vessels in the case of adrenaline may be considerable, the blood flow may be smaller than before; only a certain degree of vaso-dilation will overcome the antagonistic effect of the stronger contraction and augment the coronary blood flow. The effect of adrenaline is often complicated by the development of a back-thrust of blood into the arterial system, as happens when the contraction of the heart is very vigorous (3). When this regurgitation of blood occurs, it constitutes another factor counterbalancing the vaso-dilator effect of the drug. During the action of adrenaline two antagonistic factors are

thus at play: (*a*) the direct vaso-dilator effect, and (*b*) the indirect effects which are due to changes of the heart beat. It is therefore not possible to predict with certainty whether the coronary blood flow will be increased or decreased, even in those experiments in which the heart rate is controlled. It is known that an increase in the coronary blood flow is produced by considerably larger doses of adrenaline than those which are necessary to affect the strength of the heart beat. It is therefore not surprising that many observers who experimented on the isolated heart report a diminution of the coronary blood flow in the case of small doses of adrenaline. This diminution, however, is not due to vaso-constriction but to an increased "vaso-compression" (Fig. 6).

Fig. 6. (*a*) Effect of injection of 0·1 c.c. of 1 : 50,000 adrenaline (at the arrow). (*b*) 30 seconds later. The coronary blood flow was 27·4 c.c. before and 15·6 c.c. after the injection of adrenaline. Plate moving at reduced speed.

In the above experiments adrenaline was injected into the perfused coronary artery so that both the perfused blood vessels and the part of the heart muscle supplied by them were equally affected by the drug. On injecting adrenaline into the systemic circulation, that is on subjecting to the drug those coronary blood vessels which are left in connection with the aorta, no definite change in the hot-wire record can be noticed in the driven heart. In hearts, the rate of which is not controlled, the effect is similar to that produced by acceleration of the heart by means of stimulation with single induction shocks(4). In a few

experiments a strengthening of the contractions of the perfused area was observed.

Thus we see that changes of the cardiac contraction and of the state of the coronary blood vessels produced by adrenaline act as two antagonistic factors in their effect upon the coronary circulation. The augmentation of the coronary blood flow produced by administration of carbon dioxide is due to a co-operation of these factors. In the strongly beating heart in which the coronary blood flow is stopped during each systole carbon dioxide leads to an upward shift of the diastolic as well as of the systolic part of the hot-wire record. Thus the compression of the coronary blood vessels during systole becomes diminished, while during diastole the blood vessels become more rapidly filled and allow a larger amount of blood to flow through them on account of loss of tone. This effect can be seen in Fig. 7 in which hot-wire records obtained

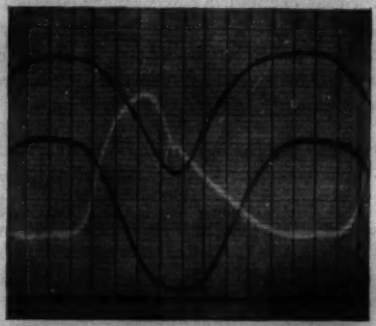

Fig. 7. (*a*) Normal record. (*b*) During the perfusion of the coronary artery with blood exposed to 7·5 p.c. CO_2. (*c*) Aortic blood-pressure. The mean aortic blood-pressure was 75 mm. and the perfusion pressure 100 mm. Hg. The coronary blood flow per cycle was 0·33 c.c. and 0·69 c.c. in (*a*) and (*b*) respectively; (*a*) is an original record, (*b*) is retraced from another plate.

before and during the action of carbon dioxide are superimposed. In this experiment blood, previously exposed to 7·5 p.c. carbon dioxide, was introduced into the perfusion reservoir and allowed to enter the left coronary artery. The mean aortic blood-pressure was maintained at 75 mm. and the perfusion pressure at 95 mm. of mercury.

The object of the experiments described in this communication has been not so much to study the action of various drugs upon the coronary

circulation as to find a method by means of which such a study could be made with a greater precision than it is possible by simply registering the inflow or outflow of blood per minute.

Summary.

The hot-wire anemometer is employed for the purpose of studying the action of chemical and pharmacological agencies on the perfused coronary system of the working heart. The method gives more precise information about the cause of changes of the coronary circulation than it is possible to obtain by other methods. Various agencies may affect the blood flow through a perfused coronary artery either indirectly by modifying the heart beat or directly by altering the tone of the coronary blood vessels. These factors are in some instances antagonistic and in others additive. Examples of the application of the method are given, and it is shown that changes in the coronary circulation at a constant perfusion pressure do not necessarily indicate simply changes in the tone of the coronary system, and that in some instances the coronary circulation may diminish in spite of a considerable relaxation of the coronary blood vessels.

I wish to express my thanks to Dr G. V. Anrep for the constant help which he gave me during the performance of these experiments.

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THE DOUBLE HEART-LUNG-KIDNEY PREPARATION.

By A. J. CANNY (*Rockefeller Fellow*), E. B. VERNEY and F. R. WINTON (*Beit Memorial Research Fellow*).

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Introductory.

The heart-lung-kidney preparation (2, 11, 9), suitable as it is for the qualitative study of the response of the kidney to a measured change in the conditions of its perfusion, becomes inadequate when it is desired to interpret such response quantitatively or to make an accurate comparison between the responses of the kidney to two stimuli which differ in their nature or intensity. The kidney during the course of its perfusion in the isolated state is gradually undergoing a functional change, a change which is reflected in an alteration with time in the degree of the response to any stimulus transitorily applied. Such is the inevitable disadvantage which is incurred through the sacrifice of a maintained normality of environment to an increased degree of control over the mechanical conditions of perfusion.

If, however, it could be shown that the two kidneys taken from the same animal and perfused with the same blood, on the one hand secreted urine at approximately the same rate and of approximately the same composition, and on the other responded in almost identical fashion to equivalent changes in their environment, then it is clear that the possibility would be presented of overcoming the disadvantage referred to above, in so far as it interferes with the accurate comparison of the responses of the kidney to stimuli differing in their nature or intensity, or with the quantitative interpretation of the response to a given change in environment. The comparison of responses simultaneously elicited would be substituted for that of responses consecutively elicited.

It is the purpose of this paper to show that these desiderata may be experimentally acquired.

A. Experimental method and technique.

Briefly the method consists in making two heart-lung-kidney preparations as closely identical as possible, with provision both for connecting the two in series so as thoroughly to mix the whole of the circulating blood, and for disconnecting them at will. The apparatus employed is shown in part in diagrammatic form in Figs. 1 and 2. The

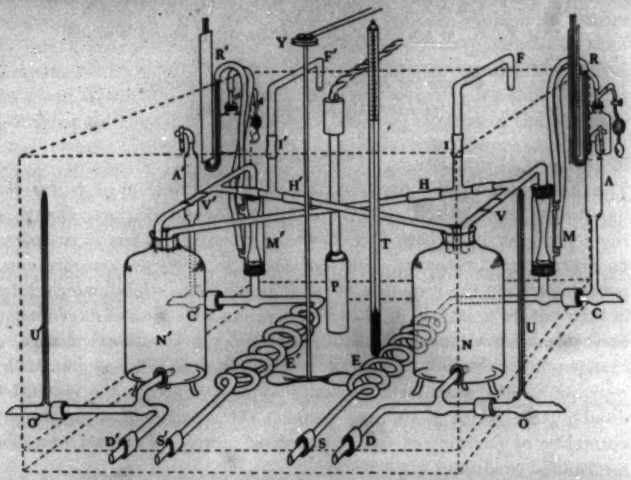

Fig. 1. Apparatus content of thermostat tank. *CC'* arterial outflow tubes from heart-lungs. *MM'* peripheral resistances. *NN'* venous reservoirs, capacity of each 700 c.c. *OO'* venous inflow tubes to heart-lungs. *SS'* arterial supply tubes to kidneys. *DD'* venous return tubes from kidneys. *HH'* cross circulating tubes for connecting the two heart-lungs in series. *Y* stirrer. *P* electric hot-point. *T* thermometer. *UU'* tubes showing the height of the blood in the venous reservoirs.

major part of the apparatus is, as indicated in Fig. 1, enclosed in a galvanized iron water-bath, the extent of which is shown by the interrupted lines.

The tube *D X D'* (Fig. 2) forms a direct connection between the two venous reservoirs, a connection which can be interrupted at any time by clamping the tube at *X*. When this tube is open the levels of blood in the two reservoirs are necessarily the same and the volumes of blood in the extra-cardiopulmonary parts of the preparations are equal. Two

dogs as nearly as possible of the same weight and build are chosen for the heart-lung preparations, and since the volumes of blood contained in the hearts and lungs are small, and their difference very small, compared with those in the rest of the apparatus, the total volume of blood circulating through the one preparation is for all practical purposes equal to that circulating through the other when the connecting tube *D' X D* (Fig. 2) is patent. The lateral pressures in the brachiocephalic cannulæ

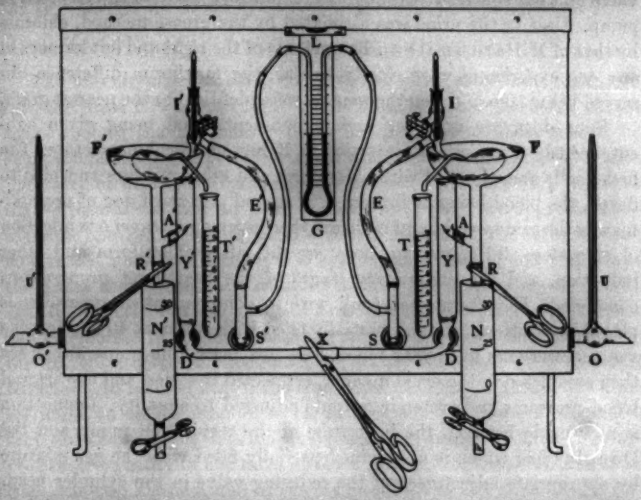

Fig. 2. Arrangement of apparatus in front of thermostat tank. *SE, S'E'* arterial supply to kidneys. *FF'* kidney receptacles. *YY'* bubble traps. *DD'* venous return tubes from kidneys. *NN'* graduated tubes for renal blood flow determinations, *OO'* venous inflow tubes to heart-lungs. *TT'* urine collecting tubes. *G* differential manometer. *L* spirit level.

are recorded by two mercurial manometers, and the pressure of supply of blood to the two kidneys can be readily brought to equality, or their difference measured, through the employment of the differential manometer *G* (Fig. 2). The temperature of the water in the tank is maintained constant by means of an electric hot-point *P* and a variable resistance, and the water is efficiently stirred by a vane *Y* driven by a small auxiliary motor.

The apparatus is placed in the centre of a long table, the two heart-

lung animals being placed on either side of it. The lungs are ventilated with 5 p.c. CO_2 in O_2 by means of Starling's respiration pump(8) of 500 c.c. capacity. The gas mixture is allowed to discharge slowly from its containing cylinder into a 40 litre Douglas bag, from which the respiration pump withdraws its charges. The gas mixture is passed through water at 40° C. on its way to the lungs of the animals, and the expired gases from each animal pass through a spear-headed rubber valve on their courses to the common expiratory valve of the respiration pump. Urea in the urine was estimated by the urease method, chloride by that of Millard Smith(5). The weights of the right and left kidneys in any one experiment were closely similar, the maximum difference observed being 10 p.c. Often the weights were identical to the nearest gram.

Four dogs are used for each experiment, each being given subcutaneously a preliminary injection of 15 mg. of morphine acetate. The first dog is anæsthetized with chloroform and ether mixture and bled to death, the blood being defibrinated. A second and third dog of approximately the same weight and build are then given an intravenous injection of chloralose, 0·1 g. per kg. body weight, after chloroform and ether induction, and the preliminary stages of the heart-lung preparations completed. The thermostat tank with its complement of apparatus is placed in position, the tank partially filled with water at 40° C., and the venous reservoirs filled with blood. The two heart-lung preparations are then completed in the usual manner, connected in series, and the arterial blood-pressures and systemic outputs adjusted to equality. Connection is now made between the inlet tube of the respiration pump and the Douglas bag which is maintained partially filled with the gas mixture by appropriate adjustment of the reducing valve in the cylinder head. Full ventilation of the lungs with 5 p.c. CO_2 in oxygen tends to maintain the reaction of the blood at its normal value by preventing the gross changes which were shown by Anrep and Cannan(1) to accompany acapnia in the heart-lung preparation.

The preparation is now ready for the attachment of the kidneys from another dog which, during the final stages of adjustment of the apparatus, has been fully anæsthetized with chloralose following chloroform and ether induction. The right kidney is excised first, after clamping its artery close to its origin and ligating the renal vein. It is perfused in the cup *F'* (Fig. 2). The left kidney is excised immediately afterwards and is perfused in the cup *F* (Fig. 2). The disposition of the kidneys as described, viz. the right kidney being placed in the left-hand receptacle and the left kidney in the right, ensures that the renal veins lie upper-

most, and so removes all risk of their patency being interfered with by the lie of the kidneys.

The preparation is now completed, the kidneys being perfused by their ipsilateral heart-lungs at the same arterial blood-pressure and with blood which, in that it is being continuously mixed, may be safely regarded at any given moment as being of the same composition in the right and left renal arteries. Measurements of the blood flow through the two kidneys are made synchronously at any desired moment.

B. Results.

The two circulations being connected in series, it is found that, as a rule, the urine flow on the right side is initially faster than that on the left, owing to the left kidney being handicapped as the result of its being connected to the perfusion apparatus a few minutes after the right. The rates of flow, however, tend gradually to approach each other, so that within about half an hour of their onset they are approximately the same and run an almost parallel course with time. The rates of blood flow through the two kidneys also approximate closely, and analysis of the urines shows that their contents in urea and in chloride are closely similar. It has been our practice to determine the percentage of urea and of chloride in the urine samples since the mechanisms involved in the excretion of these substances are widely different. It is therefore reasonable to assume that if the compositions of the right and left urine are found to be the same in so far as urea and chloride are concerned, they are the same as regards any remaining urinary solutes. The approximate equality in the rates of blood flow through, and of urine flow from, the two kidneys, and in the compositions of the urine on the two sides at constant blood-pressure, is shown in the initial periods of the experiments charted in Figs. 3 and 5. The amounts in mg. per 15 minutes of urea and of chloride secreted by the two kidneys are given in figures on the percentage graphs, and it will be seen how closely those on the one side correspond with those on the other.

At a time, then, when the two kidneys perfused at the same pressure are secreting urines closely similar in rate of flow and in composition, and when the blood flows through the two organs are also closely similar, it would be reasonable to regard them as being in the same physiological state, with the result which follows inevitably that equal responses would be given to superimposed stimuli of equal intensity. That this is so has been shown by Miss Pickford and one of us(6) to be the case in the heart-lung-double-kidney preparation, a preparation in which the

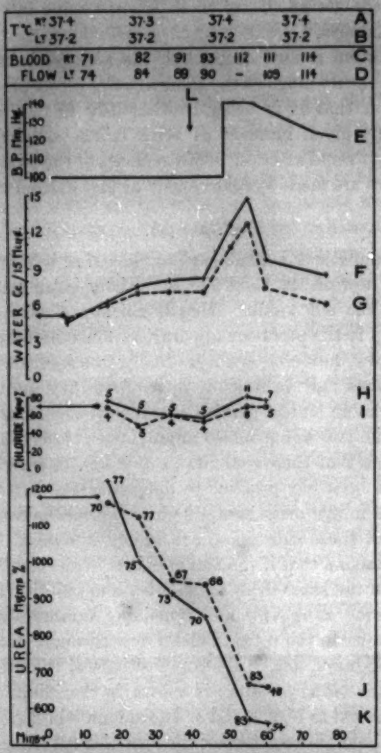

Fig. 3. Double heart-lung-kidney preparation. To show the responses of the kidney to equal changes in blood-pressure. 1 g. urea added to each circulation. Left kidney connected before right. *A* = temperature in °C. of blood in right arterial cannula, *B* = that of blood in left. *C* = blood flow in c.c./min. through right kidney, *D* = that through left. *E* = mean arterial blood-pressure in mm. Hg. *F* = rate of urine flow from right kidney, *G* = that from left. *H* = percentage of chloride (as NaCl) in urine from right kidney, *I* = that in urine from left. *J* = percentage of urea in urine from left kidney, *K* = that in urine from right. The circulations were separated at *L*. The figures on the chloride and urea graphs give the amounts in mg. secreted per 15 min. intervals of time. Post-mortem the right kidney weighed 24 g., the left 26 g. Microscopically each showed small and scattered zones of fibrosis.

two kidneys are perfused by means of a single heart-lung. In the preparation which we are now describing, it seemed possible that differences in the metabolic activity of the two heart-lung preparations might, when the circulations were separated, lead so rapidly to differences in the composition of the blood in the two circuits that the responses of the kidneys, initially equal, would speedily diverge the one from the other, so that any additional responses elicited by the subsequent imposition of further stimuli would no longer be comparable. In successful experiments, however, in which cardiac tone is good and there is no appreciable leakage of blood from either circuit, complete separation of the two circulations does not interfere with the parallelism in the activity of the two kidneys, nor with that of their responses to equal changes in arterial blood-pressure (Figs. 3, 4 and 6).

The degree of approximation reached in these experiments will be apparent from the figures in the experiments which have been quoted. It seemed advisable, however, that some numerical value should if possible be given to this approximation, and Dr E. S. Pearson very kindly suggested the manner in which the results of our experiments could be most fairly summarized.

In doing this we have omitted data from unsuccessful experiments, and from the early period of successful experiments during which urine flow was first becoming established after the inhibition usually associated with the transference of the kidneys to the artificial circulation. The average of the differences between the right and left kidneys were then taken for two consecutive samples of urine; and from this average the differences between the two sides, in the subsequent samples of any one experiment, were recorded as deviations. In order to show the degree of parallelism between the two organs at progressive stages of our experiments, we have grouped the deviations according as they corresponded to (1) the third sample of urine, *i.e.* the first collected after the two which were averaged, (2) the fourth, fifth and sixth samples, (3) the seventh and subsequent samples.

In Table I are given (I) the mean absolute values of the quantities whose differences are being considered; (II) the mean, and its standard deviation (S.D.), of the average differences of the first two samples obtained from the whole series of experiments, the differences all being counted positive and thus showing the variation of the differences among our experiments; (III) the mean and S.D. of the average differences of the chosen two samples from all the experiments, taking into account their sign, excess of right over left being counted positive; this would

indicate a systematic difference, if any, between the isolated kidneys from either side; (IV) the root mean square (R.M.S.) of the deviations of third sample differences from the average differences of the first two; (V) the R.M.S. of the deviations of fourth, fifth and sixth sample differences from the same averages; and (VI) the R.M.S. of the deviations of the seventh and subsequent sample differences from the same averages. The figures in brackets indicate the number of observations.

TABLE I. Summary of the results of those experiments in which no change was actively made in the composition of the blood.

	Blood flow c.c./min.	Urine flow c.c./15 min.	Urea in urine mg. p.c.	Chloride in urine (as NaCl) mg. p.c.
I. Mean absolute values	121 (19)	12·3 (19)	840 (18)	85 (19)
II. Mean and S.D. of differences of averaged first two samples, counting all as positive	8 ± 8 (19)	1·0 ± 0·8 (19)	76 ± 64 (18)	10 ± 10 (19)
III. Mean and S.D. of differences of averaged first two samples, counting excess of right over left as positive	−3 ± 11 (19)	+0·1 ± 1·3 (19)	−32 ± 85 (18)	+4 ± 13 (19)
IV. R.M.S. of deviations of third sample differences from average differences of two previous samples	3 (9)	1·0 (16)	30 (4)	12 (8)
V. R.M.S. of deviations of fourth, fifth and sixth sample differences from average differences of first two samples	4 (8)	1·3 (15)	48 (6)	17 (9)
VI. R.M.S. of deviations of seventh and subsequent sample differences from average differences of first two samples	3 (9)	1·3 (12)	—	19 (7)

Absolute equality in the pressure of the blood supply to each kidney can be attained with ease, and attention has already been directed to the equality in the responses which are elicited. Stimuli produced, however, by the addition of an equal amount of some chemical substance to each circulation might conceivably be unequal in intensity owing to slight differences in the volumes of blood in the two circuits, or to differences in the partition of the drug between the blood serum and the tissues of the two heart-lungs, or to both. It was necessary, therefore, to determine whether such potential differences were of sufficient magnitude to disturb the anticipated equality in the response of the kidneys to such changes in the composition of the blood perfused through them. Two drugs, to the presence of each of which the isolated kidney of the dog responds in characteristic fashion, have been employed to

control the applicability of the method to the quantitative comparison of the effects of drugs on urinary secretion. The two substances selected are caffeine and pituitary extract.

In the experiment charted in Fig. 4 the circulations were separated

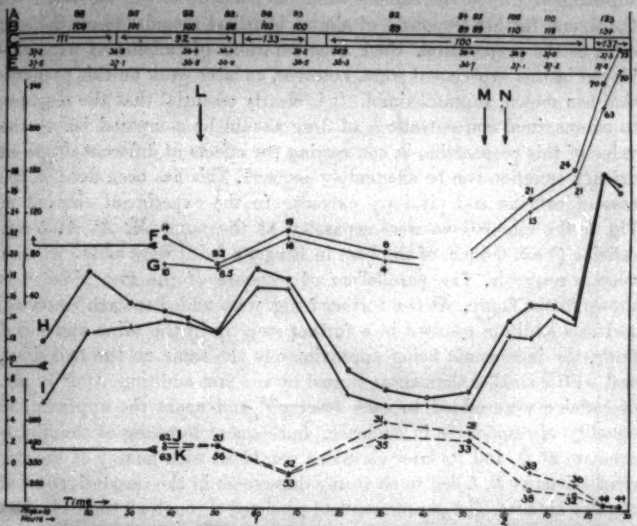

Fig. 4. Double heart-lung-kidney preparation. To show approximate equality in the responses of the two kidneys to equal changes in blood-pressure and to equivalent and supramaximal doses of caffeine citrate. 1 g. urea added to each circulation. Left kidney connected before right.

A = blood flow in c.c./min. through left kidney, *B* = that through right. *C* = mean arterial pressure in mm. Hg. *D* = temperature in ° C. of blood in left arterial cannula, *E* = that of blood in right. *F* = percentage of chloride (as NaCl) in urine from left kidney, *G* = that in urine from right. *H* = rate of urine flow from left kidney, *I* = that from right. *J* = percentage of urea in the urine from right kidney, *K* = that in urine from left. *L* and the figures on the chloride and urea graphs have the same meaning as in Fig. 3, q.v. *M*, 2 c.c. 2 p.c. caffeine citrate added to each venous reservoir. *N*, 1 c.c. 2 p.c. caffeine citrate added to each venous reservoir. Postmortem the right kidney weighed 26 g., the left 25·5 g. Microscopically the kidneys showed no fibrosis.

at the point *L*. Sixty-eight minutes later at a time when the two kidneys were still secreting urine of approximately the same volume and composition, 40 mg. caffeine citrate in 2 p.c. solution in Ringer's fluid were

added (*M*, Fig. 4) and a further 20 mg. 5 minutes later (*N*, Fig. 4). It will be apparent from the figure how closely the two kidneys responded, in so far as their blood flows, urine flows and urine compositions are concerned, to these stimuli. A subsequent rise in perfusion pressure superimposed upon the caffeine stimulus elicited, as will be seen from the figure, further responses of almost identical magnitude on the two sides of the preparation. Such concentrations of caffeine as were employed in this experiment were, however, as later work on this preparation has shown, supramaximal. It is clearly essential that the response to submaximal concentrations of drug should be compared before the value of this preparation in contrasting the effects of different drugs on urinary secretion can be adequately assessed. This has been done in the case of caffeine and pituitary extract. In the experiment charted in Fig. 5 the circulations were separated at the point *M*. At *N*, 5 mg. caffeine (1 c.c. 0·5 p.c. of the base in Ringer's fluid) were added to each venous reservoir. The parallelism of response of the two kidneys is shown in the figure. At *O* a further 5 mg. were added to each reservoir, and this addition resulted in a further step up in the urine and blood flows, the increments being approximately the same on the two sides, and a little smaller than those caused by the first addition. At *P* 10 mg. of caffeine were added to each reservoir, and again the approximate equality of response is in evidence. Subsequent lowering of the blood-pressure at *Q*, and its later elevation combined with mixing of the two circulations at *R*, failed to elicit any differences in the responsiveness of the two kidneys. The parallelism had persisted through all these changes in environment, changes which were spread over an interval of time as long as three hours.

In Fig. 6 are charted the results of an experiment in which submaximal doses of pituitary extract were added to each venous reservoir. The circulations were separated at zero time in the figure. At *L* one-fifth of a unit of pituitary extract (B. W. and Co. "Infundin") was added to each circuit, and three further additions of this amount were made at the times shown by the arrows. The urine flow from the right kidney fell gradually from 26·3 to 7·4 c.c./15 min., that from the left from 26·0 to 7·4 c.c./15 min. These were still submaximal responses since maximal doses invariably arrest the flow of urine. At *M* the circulations were connected in series: the rate of urine flow on the one side maintained its equality with that on the other.

This series of experiments, then, has demonstrated the approximate equality of the responses of the two kidneys to equivalent stimuli

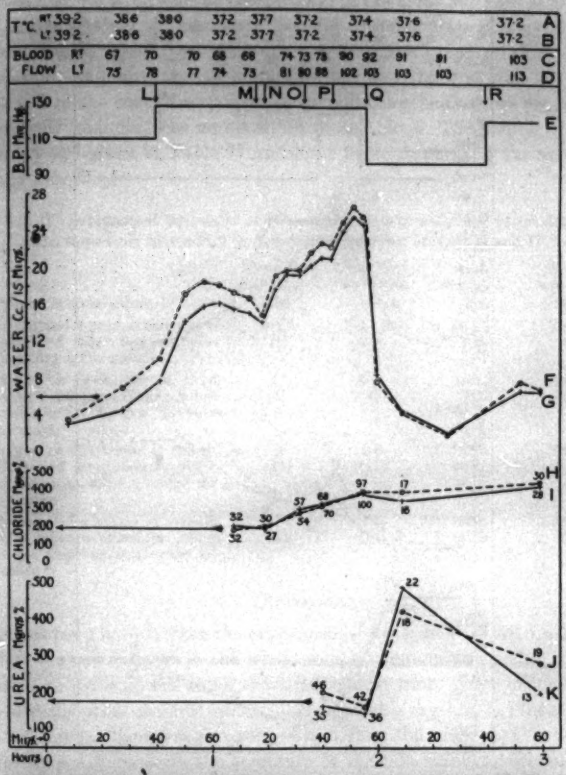

Fig. 5. Double heart-lung-kidney preparation. To show approximate equality in the responses of the two kidneys to equal and submaximal doses of caffeine. 1 g. urea added to each circulation. Right kidney connected before left. *A*, *B*, *C*, *D*, *E*, *J*, *K*, and the figures on the chloride and urea graphs have the same meaning as in Fig. 3, q.v., *F*, *G*, *H*, *I*, as in Fig. 3 but read right for left and *vice versa*. At *L* the blood-pressures were raised. At *M* the circulations were separated. At *N*, 1 c.c. 0·5 p.c. caffeine was added to each venous reservoir. This was repeated at *O*, and at *P* 2 c.c. of the solution were added to each venous reservoir. At *Q* the blood-pressures were lowered, and were raised at *R*.

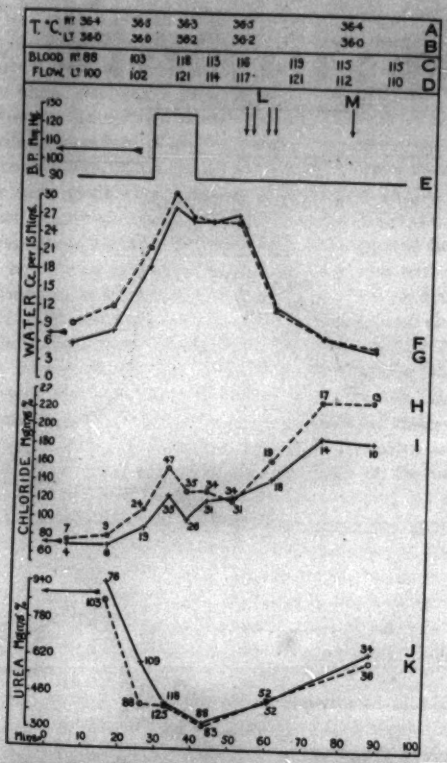
T. °C. Rt 36·4 36·3 36·3 36·5 36·4 A
Lt 36·0 36·0 36·2 36·2 36·0 B
BLOOD Rt 88 103 118 115 116 119 115 115 C
FLOW. Lt 100 102 121 114 117 121 112 110 D
L
M
B.P. mm Hg
E
WATER Cc. per 15 Mins.
F G
CHLORIDE Milligms %
H I
UREA Milligms %
J K
Mins.

Fig. 6. Double heart-lung-kidney preparation. To show approximate equality in the responses of the two kidneys to equal and submaximal doses of pituitary extract.

1 g. urea added to each circulation. Left kidney connected before right. *A*, *B*, *C*, *D*, *E*, *F*, *G*, *H*, *I* have the same meaning as in Fig. 5, q.v. *J*, *K* and the figures on the urea and chloride graphs as in Fig. 4, q.v. Circulations were separated at beginning of experiment. At *L*, 0·2 units pituitary extract (B. W. and Co. "Infundin") were added to each venous reservoir at each of the times marked by the arrows. At *M* the circulations were connected in series.

In Figs. 3–6 the time of a plotted point is the mean time of the interval during which the specimen of urine was collected.

produced by variations in the composition of the blood supplied to them. The figures relating to these responses have been collected from various experiments in which the same dose of drug has been added to each circulation, and have been summarized in a manner similar to that employed in the case of responses to stimuli other than those produced by actively changing the composition of the blood. The results of this summary are given in Table II and have been obtained in the manner already described.

TABLE II. Summary of the results of those experiments in which equivalent doses of the same drug were added to each venous reservoir between II and IV.

	Blood flow c.c./min.	Urine flow c.c./15 min.	Urea mg. p.c.	NaCl mg. p.c.
I. Mean absolute values	106	19·6	275	220
II. Mean and S.D. of differences of averaged first two samples, counting all as positive	8 ± 8 (4)	1·2 ± 0·3 (4)	38 ± 17 (4)	3 ± 3 (4)
IV. R.M.S. of deviations of third sample differences from average differences of two previous samples	1 (4)	0·9 (4)	93 (4)	20 (4)
V. R.M.S. of deviations of fourth, fifth and sixth sample differences from same average differences	4 (11)	0·9 (11)	52 (8)	28 (9)
VI. R.M.S. of deviations of seventh and subsequent sample differences from same average differences	7 (9)	1·3 (18)	40 (7)	22 (9)

C. Discussion.

It has been known since the experimental work of Goll and Ludwig (3) that the two kidneys in the whole animal commonly furnish unequal volumes of urine in the same short interval of time. First one kidney and then the other secretes more quickly, and this rhythm, as Hermann pointed out (4), disproves the assumption that the inequality is determined by a difference in the structure of the two kidneys. Hermann collected the urines simultaneously secreted by the kidneys of the dog and showed that the secretion of each as regards rate and composition of the urine might be independent of that of the other. Commonly the higher rate of flow was accompanied by a lower concentration of urea in the urine, and in explanation of the occasional occurrence of the reverse accompaniment he suggested that "die Ungleichheiten der Harnabscheidung auf beiden Nieren nicht allein in einer verschieden starken Absonderungsgeschwindigkeit auf der Flächeneinheit begründet sei, sondern auch daher rühren könne, dass die Niere nicht zu allen

Zeiten auf ihrer ganzen Fläche Harn abscheide." These theoretical considerations of Hermann were given a concrete basis in fact by the experiments of Richards and Schmidt(7) in which intermittence of the glomerular circulation was observed directly in the frog's kidney.

Reference has already been made to the equality in the weights of the two kidneys. Equality in the rates of urine flow is not, however, invariably obtained with this preparation at equal arterial blood-pressures. Sometimes considerable differences are observed and persist throughout the experiment. But in such cases the percentile content in urea of the urine secreted at the slower rate is, so far as our observations have gone, invariably greater than that of the urine secreted at the higher, whilst the amount secreted per unit interval of time is smaller. When the rates of urine flow are the same on the two sides the compositions of the urines, as has been shown in this paper, approximate closely.

It would seem probable that the inequality in the rates of urine flow, when encountered, is due simply to a fall in the effectiveness of the pressure stimulus to the more slowly secreting kidney, a fall which is *uniformly* distributed to the units of which the kidney is composed. If this be the case, reduction in the pressure of the blood supply to the more rapidly secreting kidney to such a degree that the rate of urine flow becomes equal to that of the other kidney, should be accompanied by a change in the composition of the urine to equality with that of the initially more slowly secreting kidney. It is hoped shortly to test this point experimentally on the heart-lung-double-kidney preparation(6, 10). But it would appear from experiments already performed and from the results of Hermann's work(4), that the composition of the urine from the isolated kidney bears a much closer relationship to the rate of secretion than does that of the urine from the kidneys *in situ*. Moreover, any rhythmic changes which may occur in the rate of urine elimination are so small as to be imperceptible. These facts suggest that the renal units are exhibiting a much more uniform activity when the kidney is being perfused in the isolated state than when working in an environment more nearly approaching the normal; and in this connection the observations of Richards and Schmidt to the effect that the number of active glomeruli seen in a given optical field in the frog's kidney is increased by section of the splanchnic nerve, and that the intermittence of the glomerular circulation can be increased by stimulation of the sympathetic supply to the kidney or by the intravenous infusion of small amounts of adrenaline, would seem to be not without interest and significance.

The rate of blood flow through the one kidney at unequal rates of urine flow and equal blood-pressures may be equal to, greater, or less than that through the other kidney. This observation, emphasizing as it does the essential independence, now commonly accepted, of urine flow and blood flow, is clearly not irreconcilable with the belief that the proximate stimulus to the secretion of urine is the intraglomerular pressure.

Summary and conclusions.

1. A method is described by which the two kidneys of a dog may be perfused by two heart-lung preparations. The circulations may either be connected so that blood of the same composition is supplied to each kidney, or separated for the purpose of accurately determining the effects on the kidney of a given change in the composition of the blood.

2. The two kidneys perfused at the same blood-pressure secrete urines which approximate closely in rate of flow and in composition.

3. Their responses to equivalent changes in environment as produced by equal changes in the arterial blood-pressure, or by the addition of equal and submaximal doses of caffeine or of pituitary extract to each circulation, are closely similar.

4. The experiments described in this paper point to the two kidneys so perfused being in approximately the same physiological state, and so to the legitimacy of expressing quantitatively the effects on the kidney of any given change in the conditions of its perfusion.

The expenses of this research were defrayed in part by a grant to one of us (E. B. V.) from the Government Grant Committee of the Royal Society, to whom we wish to express our best thanks.

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OBSERVATIONS ON THE STANDARDIZATION OF THE WATER-SOLUBLE, ŒSTRUS-PRODUCING HORMONE.

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It is not proposed to enter into a detailed historical summary of the many investigations that have been performed on the standardization of the œstrus-producing hormone, as this subject has been fully reviewed by Allen and Doisy(1). The salient points may be noted as follows.

Firstly, the application by Allen and Doisy(2) of the Stockard and Papanicolaou(3) smear method to the study of the problem. The unit of the material was estimated by finding the minimum quantity capable of producing œstrus when injected into a rat. Whilst Allen and Doisy gave the injection in three portions spread over the first 12 hours, later workers used a single injection but still adhered to the method of giving a series of diminishing doses to a few animals, and determining the least amount that would give a positive result.

Standardizations on these lines were severely criticized by Coward and Burn(4) who pointed out that rats are irregular in their response to the material. On the basis of a more general discussion by Trevan(5) of the errors in methods of this kind, they evolved a method for standardizing the oily material, in which they employed a large number of rats and recorded the results statistically. By this method the unit was defined as that quantity of material capable of producing œstrus in 50 p.c. of the rats used. In this paper Coward and Burn considered that a single dose is as effective as a divided one, but Burn(6) has recently stated that this is not the case with the water-soluble material.

Laqueur(7), working with water-soluble material, maintained that the administration of the hormone should be spread out over a period of 36 hours. Allan, Dickens, Dodds and Howitt(8) reported experiments supporting the view of Laqueur, and it was shown that water-soluble

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material made by their process was relatively inactive when given in one dose, but was highly active when the same quantity of material was given in doses spread over 36 hours. In this paper it was pointed out that the work of Coward and Burn would have to be repeated because these observers had worked exclusively with the oily preparation, and it became evident from our earlier observations, such as that mentioned above, that the water-soluble material does not obey the same laws.

As then pointed out, it was thought necessary to construct a curve on lines similar to that of the above workers, plotting along one co-ordinate the amount of material injected, and along the other the number of rats showing œstrus. There is another factor to take into consideration, namely the distribution of the dose. The object of the present communication is to report the results of carrying out these suggestions and to establish the relations between the amount of material injected, the spacing of the injections, and the number of rats showing œstrus.

The fact that water-soluble material is much more active when given by spaced injection has been confirmed by Marrian(9) and by Burn(6). Kahnt and Doisy(10) discussed the original work of Allen and Doisy in the light of the recent publications referred to above. It is difficult, however, to gather from their paper whether they are definitely abandoning the method of the diminishing dose in favour of the statistical technique.

Laqueur and de Jongh(11) have recently described a method of preliminary assay, using batches of three mice. Each group of three animals receives a different dilution of the unknown material. For example, it may be found that in one group two animals are positive and one negative, whereas in the group receiving a slightly larger dose all three are positive. The unit is, therefore, assumed to lie between the doses received by these two groups. This may then be confirmed by an assay on twelve or more animals, each of which receives the same dose. The preliminary portion of the above method is similar to the original diminishing dose technique, extended by the use of three animals per dose instead of one. It seems, therefore, to be open to the same objections as its predecessor, and from our own experience with the rat, it seems likely to lead to contradictory and confusing results.

It is clear that the effect produced by the hormone is closely related to the rate at which it is absorbed, and it is to be expected that, by giving the material emulsified with oil, the rate of absorption would be decreased. In this way the oily material may be considered as constituting a store or depot of hormone from which the animal continuously receives small

quantities. This method may, therefore, be expected to approach much more nearly to the natural process. The relationship to the absorption is shown in the following experiment where, by injecting the previously standardized aqueous material in the form of a single dose of an oily emulsion, the activity is much increased as compared with the effect produced by a single injection without the added oil.

We have also obtained some quantitative data concerning the response to the œstrus-producing hormone when given to rats by mouth. It was thought that the negative results previously reported, following the administration of moderate amounts of hormone, by this route, might be due to a similar cause to that giving negative results from a single subcutaneous dose. In view of this, doses were given by the mouth at 3-hourly intervals for 36 hours, but in this case also, large doses were necessary to obtain a positive result.

Experimental.

Animals. For the following experiments rats, obtained mainly from the Glaxo Laboratories, have been used. The animals were ovariectomized when they reached a weight of 140 g., the operation being performed by the lumbar route. Care was taken to remove with the ovaries all the surrounding fat, the Fallopian tube, and the upper portion ($\frac{1}{2}$ in.) of each horn of the uterus. The fact that ovarian regeneration in the rat has, in our experience, been exceedingly rare, is probably accounted for by this complete removal of the fatty tissues surrounding the ovary. In the case of the mouse there is evidence that regeneration is comparatively common. Parkes(12) has observed that regeneration occurs in about 6 p.c. of the animals used by him. From our results, based on nearly 1000 ovariectomies, it is probable that with the rat the percentage is much lower—it is certainly not more than 0·3 p.c.

We would emphasize the necessity for observing great care in regard to asepsis during the operations. Although the large majority of the wounds heal by first intention even when little regard is paid to asepsis, post-mortem examination of such animals shows that small abscesses may be present at the site of ligation of the uterus. A discharge of leucocytes from this source leads to their appearance in the vaginal smear at all stages of the cycle. Further, the occurrence of a pyometra has in two instances led to the appearance of smears nearly approaching the œstrus smear in the ovariectomized, uninjected animal.

Use of animals. The animals used in routine testing work are injected every two weeks. A second group of animals, used chiefly for experiments

on standardization, has on some occasions been used for several consecutive weeks without any abnormality in the results being observed. This is in fact only to be expected, for the last smear from most of the animals used in any test shows a marked tendency to return to a complete dioestrus. In view of Kahnt and Doisy's experience with regard to "priming" the animals, it would even seem to be advantageous to use the animals every week.

Diet. The diet was calculated on the basis of 15 g. of food per 100 g. rat weight, made up in the proportions: protein 3 g., fat 2 g., carbohydrate 10 g. It consisted of bread and milk with the addition of margarine, casein, marmite and oatmeal. On this diet the unovariectomized female maintained a regular cycle and weight.

Technique of standardization. The method which was described in an earlier paper(8) has in the main been followed in the present series of experiments. Where six injections of the material under test were given to each rat, the following were the times of administration:

(1) First day 9 a.m. and (2) 5.30 p.m.

(3) Second day 9 a.m., (4) 3 p.m., and (5) 9 p.m.

(6) Third day 9 a.m.

The injections were made with a calibrated tuberculin 1 c.c. syringe, graduated to 0·01 c.c. The dose given has been 0·2 c.c. throughout and all preparations are diluted accordingly before injection. It has become clear, however, that an increased accuracy is attained by increasing the volume of the individual injections, without any marked effect on the response being observed. For all future work, therefore, we shall use 0·5 c.c. per injection.

Vaginal smears. We have found that in rats the character of the vaginal smear changes very quickly. This makes it possible to miss the stage of complete cornification if smears are only taken in the morning and the evening. A normal rat with intact ovaries, if smeared every hour throughout the day, may show several smears containing only cornified epithelium, followed shortly after by the appearance in one or two smears of a number of leucocytes. Later on the leucocytes are not found, and the smear returns to the full œstrus type. Thus it is advisable to take smears frequently in order to be certain that the maximum reaction is observed. Smears have, therefore, been taken three times on the last day, making seven smears in all, viz.:

(1) First day.

(2) Second day.

(3) Third day 9.30 a.m.

(4) Third day 5.30 p.m.

(5) Fourth day 9 a.m., (6) 2 p.m., (7) 5.30 p.m.

The last three smears are taken into a drop of water on the slide and are well stirred, thus producing a thin film. All the smears are then stained with hæmotoxylin and eosin.

Evaluation of the smears. With young and little used rats the œstrus smear consists of cornified cells only. By spreading in a drop of water each cell can be clearly seen distinct from the others, and the presence of even a few leucocytes is easily detected. When the animals have been in use for some time the clean type of smear is no longer obtained, and instead the field is obscured by quantities of mucus. This not only binds the cells together in clumps, but also tends to take up the stain, thus rendering accurate observation difficult. A drop of liquid paraffin spread over the whole slide assists in distinguishing between leucocytes and deeply stained mucus.

In recording our experimental results, for reasons discussed below, we have been compelled to recognize two types of smear. The clear type of œstrus smear, containing cornified cells only, we have called œstrus + (*E* +). The mucus-containing type, if on careful examination it proves to contain only cornified cells, also comes under the heading œstrus +. Smears containing in addition to fully cornified cells a very small percentage of leucocytes and a few nucleated epithelial cells, we have called œstrus − (*E* −). Both the above types must be clearly distinguished from the strongly pro-œstrus smear in which leucocytes and nucleated epithelial cells are present in a larger proportion, perhaps up to 20 p.c. of the total. Between the pro-œstrus and œstrus smears a gradation of intermediate types is observed containing varying proportions of leucocytes and nucleated epithelial cells.

Up to this point the method is simple, but it is in the interpretation of the results that the great difficulty is encountered. Whilst it is easy to state that œstrus is that stage of the cycle where there are only cornified cells present in the vaginal smear, the observer is at once faced with the difficulty of assessing the value of a smear that does not quite reach this standard.

It is obviously wrong to dismiss a preparation as inactive when the smears contain in addition to fully cornified cells a very small percentage of leucocytes and a very few nucleated epithelial cells. Again, in the case of the rat, the natural œstrus occurring in the unovariectomized

animal often falls short of what is regarded as a typical full œstrus. Theoretically each type of smear should receive a definite value and should contribute its quota to the total value assigned finally to the material under test. Loewe(13,14) has described a method using a somewhat similar principle, but to apply this to our method it would be necessary to fix an arbitrary standard with which the smears from each rat could be compared, the actual percentages of each type of cell being determined by counting. This is almost impracticable on a large scale, not only because of the amount of labour involved, but also because of the difficulties introduced by the presence of mucus. In view of these difficulties, it is best to make use of those responses on which a clear-cut verdict can be given, namely œstrus + and œstrus −, and to rank all other types of smear as negative.

Results.

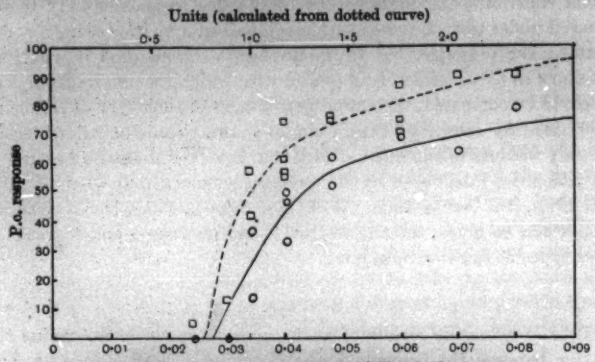
Table I gives the results of an experiment made to determine the relation between the percentage of rats giving a positive response and

Table I. Batch No. 364.

Number of animals	Smear		Total response	Original material
	E+	*E*−	p.c.	c.c.
20	14	3	85	0·075
23	9	7	69	0·06
16	11	1	75	0·06
23	11	6	73·8	0·06
23	11	6	73·8	0·06
16	11	3	87	0·06
16	10	2	75	0·048
23	17	2	82·5	0·048
21	7	6	62	0·04
16	8	4	75	0·04
21	10	2	57	0·04
16	8	1	56·2	0·04
21	9	7	76	0·048
24	16	4	83	0·048
21	3	6	43	0·034
24	9	5	58	0·034
19	1	3	21	0·034
22	14	3	77	0·06
45	—	3	7	0·024
31	22	5	87	0·06
23	8	5	57	0·06
29	16	8	82	0·06
48	47	—	98	0·092
39	25	10	90	0·071
39	31	4	90	0·080
39	0	5	13	0·030

the total dose given. The results have been given in two forms, first taking only œstrus +, and secondly œstrus + together with œstrus −. From

the curves (Fig. 1) it will be seen that, as would be expected, a larger quantity, namely 4·4 as against 3·4 hundredths c.c., is required to



Full curve and circles: œstrus + only. Dotted curve and squares: œstrus + and œstrus −, total response.

Fig. 1.

produce a complete cornification in 50 p.c. of the rats used. On the basis of these curves two methods suggest themselves, namely, to take œstrus + only, or to take the œstrus + and œstrus − curve. It will be seen that the œstrus + curve is perhaps the more regular. In spite of this fact we have adopted the total positive (œstrus + and œstrus −) curve for the following reasons: firstly, as has been mentioned above, the smear from the normal, unovariectomized animal frequently falls short of the œstrus + standard, and secondly, the œstrus − smears are so little removed from the full œstrus type that it seems unreasonable to ignore them in assessing the activity of the preparation used. Whether the smear is œstrus + or œstrus − seems likely to be due to variations such as are encountered on taking repeated smears from the same animal (see p. 351) or to transitory irregularity in the individual rats. In either case such slight variations could have little significance in estimating the activity of the preparation.

These curves thus give a method whereby any unknown preparation may be compared with the standard one. On the other hand, assuming that all water-soluble preparations behave in a similar manner, and that the over-all response of the rats remains sensibly constant, the dose

necessary to produce a given percentage response may be calculated from any individual determination by reference to the curve.

We have followed Coward and Burn in taking as one unit the amount of material required to give an œstrus response in 50 p.c. of the animals. The calculation of the amount of one unit by reference to the curve cannot be made with any accuracy if the observed response is positive in below 35 p.c. or above 70 p.c. of the animals.

In our opinion it is essential to kill off at once any animal showing the slightest sign of disease. Amongst our animals lung infections have occasionally been observed. In contrast to Laqueur's experience with mice(11), we have found that the infected animals do not die suddenly, but only after a variable period of ill health. During this period, when the animals may not show any very marked signs of disease, they do show a pronounced decrease in sensitiveness. For example:

Old rats in ill health; 12 months since ovariectomy.
Batch No. 364 diluted × 35. Six doses of 0·2 c.c. (routine method).
No. of rats 19. Result 1 *E*, 3 *E* − =4/19 (*i.e.* 21 p.c.).

Young and healthy rats; six weeks since ovariectomy. Same material, same dose.
No. of rats 21. Result 3 *E*, 6 *E* − =9/21 (*i.e.* 43 p.c.).
No. of rats 24. Result 9 *E*, 5 *E* − =14/24 (*i.e.* 58 p.c.).

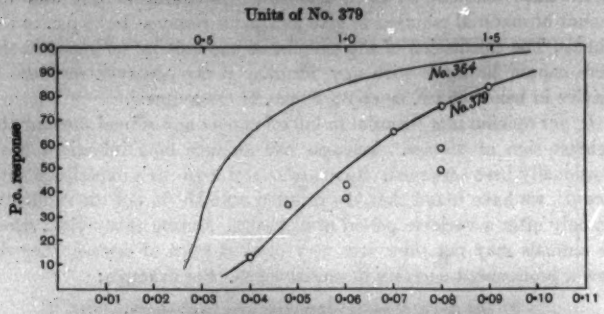
With this reservation the response to two different preparations made by the same method follows much the same course, even if different rats are used. Table II gives the results obtained with another batch of hormone. The relation between the response to these two batches (Nos. 364

Table II. Batch No. 379 (six doses of 0·2 c.c. diluted material).

Number of animals	Smear		Total response p.c.	Original material c.c.
	E+	*E*−		
23	5	3	34·7	0·048
19	8	3	58	0·08
24	19	5	100	0·12
23	1	2	13·0	0·04
22	6	8	63·6	0·07
24	18	2	83	0·09
24	2	7	37·5	0·06
23	1	9	48	0·08
24	10	8	75	0·08
23	3	7	43	0·06

and 379) is not at once clear from Fig. 2 in which the actual doses are plotted against the percentage response, but if the amounts given are calculated in terms of the unit (50 p.c. response) dose, the result (Fig. 3) shows that the agreement is satisfactory. Individual variations, however, are very great, and an assay based on a single determination is liable to

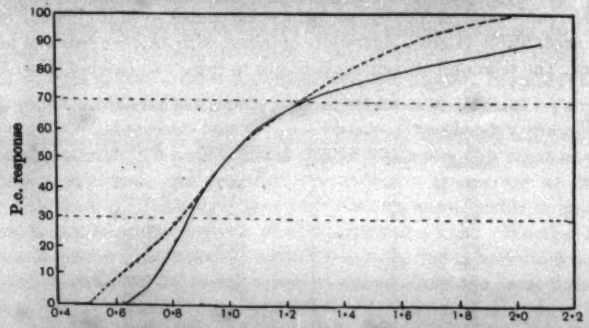
a considerable error even if 24 rats are used. Thus the use of a standard preparation, as first suggested by Laqueur, has much to recommend it,



Total dose in c.c. undiluted material (given as six doses over 48 hours).
Upper curve Batch No. 364 (dotted curve of Fig. 1); lower curve Batch No. 379.

Fig. 2.

provided that several dilutions of the standard and the unknown are compared, using groups of about 24 rats for each dilution.



Total number of units injected. Dotted curve = No. 379; full curve = No. 364.

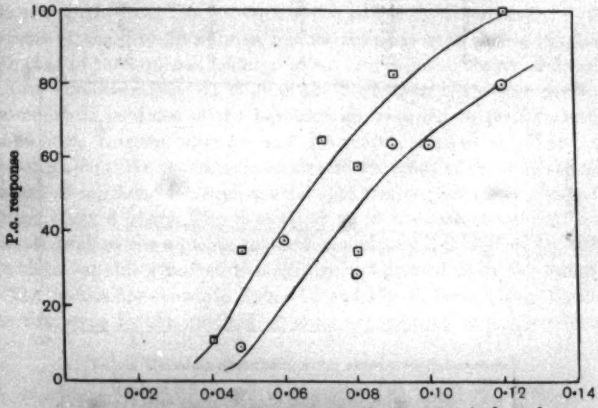
Fig. 3.

The preceding results were obtained by using the method detailed above, namely by the use of six doses given as described. The effect of other methods of administration was now investigated, and first the

effect of dividing the dose into four parts only, as recommended by Marrian(9), was studied. The same batch of material, No. 379, was injected into the same rats on different occasions, using both methods. The results are shown in Table III and are graphically represented in Fig. 4. The marked difference, amounting to an approximately 25 p.c. greater response from the administration of six doses, can only be attributed to a difference in the amount of hormone utilized by the animal, and consequently is related to the absorption and loss either through destruction in the body or by excretion.

TABLE III. Batch No. 379 (four doses of 0·3 c.c. diluted material).

Number of animals	Smear		Total response p.c.	Original material c.c.
	E+	*E*−		
23	1	1	9	0·048
24	1	8	37·5	0·06
21	6	0	28·5	0·08
24	12	8	80	0·12
22	6	8	64	0·09
22	5	9	64	0·09
22	6	8	64	0·10



Total dose in c.c. of No. 379. Upper curve and squares: as six doses; lower curve and circles: as four doses (Table III).

Fig. 4.

Single dose method. In the above experiments the absorption of the aqueous material is probably constant, and the difference in the results must be ascribed to a greater loss by destruction or elimination in the

case of the four-dose administration. An extreme case of this effect is provided by the administration of a single dose containing the same total number of units as was previously given in the form of divided doses. Here the high concentration of hormone in the blood and tissues rapidly produced by this method of administration is presumably followed by rapid excretion or destruction. It is known that the hormone is readily excreted by the kidneys, as is shown for instance by its presence in large amounts in the urine during pregnancy (15), but there does not seem to be any direct proof that this is the fate of the hormone when administered subcutaneously.

The results of injecting single doses, containing a varying number of units as estimated by the routine method of six injections, are given in the following table and curve.

TABLE IV. Single doses of aqueous material: "total units" determined by routine method of 50 p.c. response to six doses.

Total units given in 1 c.c.	Number of animals	Smear		Total response p.c.
		E+	*E*−	
1	15	0	0	0
2	14	0	0	0
5	23	1	1	9
20	24	1	2	12·5
50	23	5	9	61

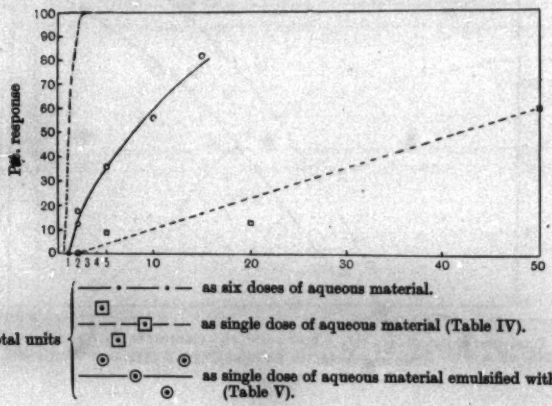


Fig. 5.

It will be seen that about 40 units are required to cause a 50 p.c. response in this case.

In order to test this hypothesis further we have attempted to delay the absorption of single doses of the hormone by administering it in the form of an emulsion prepared by shaking the aqueous material with suitable amounts of olive oil and traces of sodium oleate. The results of such experiments are shown in Table V and Fig. 5. The emulsion used contained 50 p.c. oil.

TABLE V. Single dose of emulsion of previously standardized aqueous material in 50 p.c. oil: "total units" by routine method of 50 p.c. response to six doses.

Total units given in 1 c.c.	Number of animals	Smear		Total response p.c.
		E+	*E*−	
1	16	0	0	0
2	24	2	1	12·5
2	16	0	3	18·5
5	22	2	6	36
10	23	5	8	56·5
15	23	11	8	82

It will be seen that here only eight units are required to produce a 50 p.c. response, as compared with 40 units for the aqueous material given also as a single dose. The presence of the oil has thus increased the effectiveness of the dose five times, but its action is nevertheless much less than that of the aqueous material given as a series of six small doses.

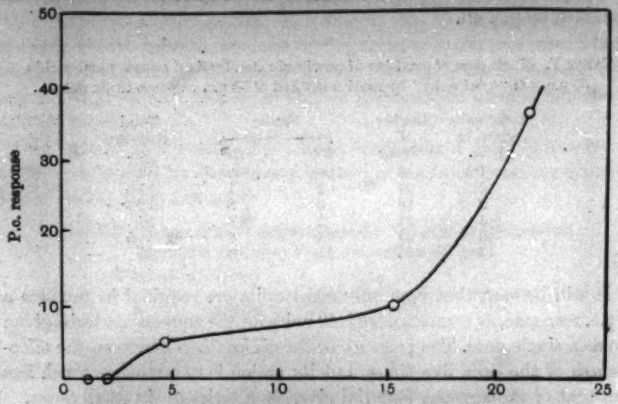
Oral administration. It is generally recognized that when given by the mouth large doses of the hormone are required to produce œstrus (Loewe(16), Loewe, Lange and Faure(17), Laqueur, Hart and de Jongh(18)). We therefore investigated the effect of dividing the total amount of hormone for each animal into twelve doses and giving one portion every 3 hours. This is easily done in the case of the rat, as the animals swallow the aqueous material completely if it is given by means of a blunt cannula attached to a syringe and pushed down the throat.

The results are shown in Table VI and Fig. 6, from which it will be seen that even by this method 20 units are required to produce œstrus

TABLE VI. Oral administration of water-soluble hormone. Twelve doses of 0·2 c.c. each at intervals of 3 hr. over 36 hr.: "total units" by routine method of 50 p.c. response to six doses.

Total units given	Number of animals	Smear		Total response p.c.
		E+	*E*−	
1	23	0	0	0
2	22	0	0	0
4	20	0	1	5
16	20	1	1	10
22	22	5	3	36

in 25 p.c. of the animals. This result seems to show that the hormone is either absorbed inefficiently or is destroyed in the alimentary canal. On account of the known stability of the hormone to acid, alkalies and digestive ferments, the latter explanation seems the less probable one.



Total units: oral administration as twelve doses of 0·2 c.c. each.

Fig. 6.

Discussion.

The curves shown in Fig. 2 were obtained by experiments extending over a considerable period of time. Thus, the first determination was made on 12. xi. 28, and the last on 25. vi. 29, a period of roughly six months. The fact that a series of points was obtained through which it is possible to draw a curve indicates that there has been no diminution in the sensitiveness of the rats, or in the activity of the material. If the points had been determined in sequence, *i.e.* small doses followed in succeeding weeks by larger and larger ones, it would have been possible for a decrease, either in the activity of the material or in the sensitiveness of the rat or both, to have occurred without this deterioration becoming obvious. The determinations were actually made in an irregular manner, so that if either rats or material had deteriorated, it would at once have become obvious by the percentage response becoming definitely smaller than previous estimates in the same neighbourhood.

The agreement between the two curves of Fig. 2, when plotted with

units as abscissae and percentage response as ordinates, indicates first, that the reaction of the rats to different materials follows much the same course; secondly, that the definition of a positive reaction which has been adopted leads to concordant results; thirdly, that different batches of rats maintain a constant sensitiveness over considerable periods of time; and fourthly, that the sensitiveness of these groups is approximately equal.

The occurrence of a few points deviating very considerably from the curve, as in Fig. 2, indicates clearly that single determinations are liable to error. It is, therefore, desirable that the assay of a new and unknown sample of material should include (*a*) one or more preliminary standardizations on a small number of rats (about eight), which will indicate approximately the dose required to bring the response within the limits of 35 p.c. and 70 p.c., and (*b*) at least two determinations on a larger number of rats (about 20) with different dilutions of the material, so arranged that the response elicited falls within the limits mentioned above, viz. 35 p.c. and 70 p.c. For the evaluation of the material in terms of units, reference must then be made to a standard curve previously constructed from some other material.

For these standardizations we have used the method of six doses, as this is the one of which we have most experience. Burn(6), however, suggests that the single dose method might equally well be used for the standardization of the aqueous material. The advantage of this method apparently lies in the smaller amount of labour involved in the standardization, but against this must be considered the great extravagance of such a procedure. As will be seen from our results (Fig. 5), a single standardization upon 20 rats would require at least 1000 units, or the total quantity from 5 kilos of placenta(8). Moreover, there are serious theoretical objections to this method. The administration of such large single doses is liable to lead to very considerable loss through incomplete absorption and through destruction and elimination, and it seems very probable that such variable factors will introduce a greater error into the results of a single injection. In any case, a satisfactory curve for the response to a single dose of aqueous material has yet to be constructed before this method could be used.

Summary.

1. Certain difficulties arising in the standardization of the œstrus-producing hormone are considered, namely:

(*a*) Evaluation of vaginal smears.

(*b*) Mode of administration of the hormone in respect to spacing of the doses.

(*c*) Variations in the response of different groups of animals to the same and to different batches of material.

2. The above problems have been studied by the construction of a series of curves showing:

(*a*) The relationship between the dose of the hormone administered and the percentage of rats showing a positive œstrus response. These curves include experiments in which different criteria of œstrus are taken, and they also show the effect of different materials on various groups of rats. These groups show that comparable results are obtainable.

(*b*) The effect of administering the active material in a single dose emulsified with oil, and

(*c*) In a series of doses given by mouth.

3. The application of these experiments to the question of standardization is discussed, and a method for routine use is described.

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THE INFLUENCE OF EXCISION AND TREATMENT WITH SALINE ON THE PACE-MAKER OF THE FROG'S HEART.

By J. J. IZQUIERDO (*Fellow of the Rockefeller Foundation*).

(*From the Physiological Laboratory, Cambridge.*)

In the course of some experiments on the effect of temperature on the rate of beat of the frog's (*Temporaria*) heart it became evident that too little attention had been paid by previous workers to the variations which take place in the pulse rate of an excised heart kept under approximately constant conditions. By the phrase "approximately constant conditions" is meant, constant temperature of a medium which is altered in composition only so far as it is affected by the heart itself.

The present paper sets further an attempt to trace the alterations which take place in the heart rate from the time the heart is in the living frog, embracing the process of excision and immersion in saline solution, and extending in some cases over periods up to one day.

When a frog's heart is placed in Ringer's solution three main periods may be distinguished so far as the rhythm of the pace-maker is concerned. (i) A period of acceleration, (ii) a period of slowing, (iii) a period of constancy.

Period (i). Usually the pulse rate immediately after it has been placed in Ringer's solution is much faster than, perhaps twice as fast as, it was in the living frog.

Period (ii). For some time after the heart (or sinus) is placed in Ringer its pace acquired in Period (i) gradually slackens. The retardation may continue for two hours or more, or on the other hand it may only last half an hour.

Period (iii). After Period (ii) the heart may beat in Ringer's solution at a very steady rate for many hours, with a slight but appreciable tendency to quicken. It is not until Period (iii) has been reached that experiments can properly be undertaken on the effect of alteration of conditions in the pulse rate, unless these alterations produce immediate and large alterations of rate.

My experiments deal only with the rate of the pace-maker which

may be as great in the third period as in the normal heart. That is not to say that the heart is normal, nor is it a criticism of the general conception of the hypodynamic heart as put forward by Clark(1). The term hypodynamic as its etymology implies deals with factors other than the rate at which the heart beats.

The following gives a more detailed account of the observations made.

Period (i).

Consists of three principal operations:

(*a*) pithing the frog with possible stimulation of the cardiac nerves;
(*b*) cutting out the heart, *i.e.* possible trauma;
(*c*) alteration of the medium from blood to saline solution.

(*a*) *Pithing* was carried out by the usual procedure. The observations

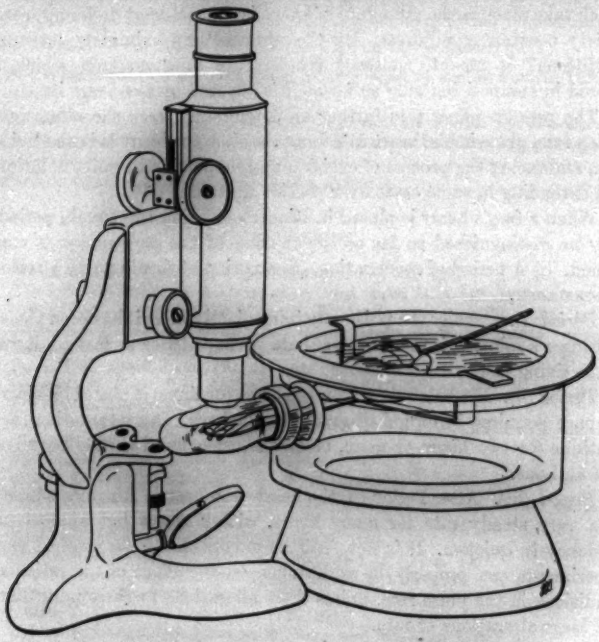

Fig. 1. Apparatus for counting the pulse rate in the intact frog.

on the rate of the pulse were made by counting the number of pulsations in an arteriole of the web of the foot.

The apparatus used for doing this was as follows (see Fig. 1). A glass chemical desiccator of the type fitted with a stopper in the side of the lower portion was filled almost to the brim with water. In place of the stopper was a tube through which the leg of the frog projected, the body, dorsal side up, being in the vessel and the nose usually above the surface of the water. The side tube was of oval section, the top and the bottom being flat, so that the web of the foot could easily be observed with the microscope and the pulsations in the arterioles and capillaries counted. The observed foot was fixed to a celluloid plate by a very fine wire attached to the toes. Some practice was necessary before a suitable method of fixing the frog with a bandage was discovered. The bandage, 4 cm. broad, was started just above the foot of the unobserved leg, the ankle was flexed so that the foot lag alongside the lower leg with the toes pointing to the knee and the leg bounded alongside the body with the ankle as near the head as possible. The whole with the anterior limbs being enclosed in the bandage which also covered the eye. The bandage was then continued for the purpose of strapping the frog to a celluloid strip of the length of the animal and mounted on a wire frame somewhat the shape of a spoon, the body being attached to the broad part and the observed leg to the part corresponding to the handle. This latter attachment was effected with a separate bandage about 2 cm. wide and finally the toes were attached to the celluloid with fine cotton-covered wire which passed round the tip of each and then round the ankle.

No further attachment is necessary than that the portion of the stand to which the observed leg is attached should be slipped into the observation tube.

If also the eyes are covered by the gauze the frog remains quiet and the frequency of the pulse usually becomes regular. In the few cases in which it did not the frog was discarded.

The following are four sample sets of observations before and after pithing, showing the variety of effects obtained.

Pulse rate before pithing	Pulse rate after pithing
22–26 (4 readings)	40–42 (5 readings in 12 minutes)
21–24 (3 „)	30–32 (4 „ 10 „)
30–31 (4 „)	Stopped for 1 minute and then 20–31 progressively in 10 minutes
27–28 (3 „)	50 dropping progressively to 29 in 30 minutes

(*b*) *Removal from the body.* To elucidate the part, if any, played by mechanical stimulation inflicted upon the heart during its isolation the following experiments were performed.

After counting the rate of the exposed heart for several minutes in urethanized frogs, one of the aortæ was, or both were, severed and the blood thus shed was pipetted off and placed in the paraffin moist-chamber (to be described below). The pipette and the chamber had been previously dusted with a few particles of heparine. This done the heart was isolated and placed in the heparinized blood. The following figures showed that the effect of cutting out the heart or placing it in the chamber for observation was in all cases small in amount and inconstant in sense.

Rates during the 15 minutes before bleeding and isolation	Rates during the first 20 minutes after isolation and in heparinized blood
23–24 (4 observations)	20–23 (5 observations)
28–33 (5 „)	30–22 (getting slower)
24–26 (4 „)	27–28 (4 observations)
36–32 (4 „)	36–28 (5 „)
30–34 (4 „)	31–28 (5 „)

(*c*) *Transference to Ringer's solution.* When however the heart is taken from the frog in precisely the way mentioned above and placed not in its own heparinized blood but in Ringer's solution—(NaCl 0·65 p.c.; KCl 0·020 p.c.; $CaCl_2$ 0·025 p.c.; $NaHCO_3$ (anhydrous) 0·015 p.c.) brought to desired *p*H[1] by addition of concentrated NaH_2PO_4 solution —then a considerable acceleration is observed as a constant phenomenon.

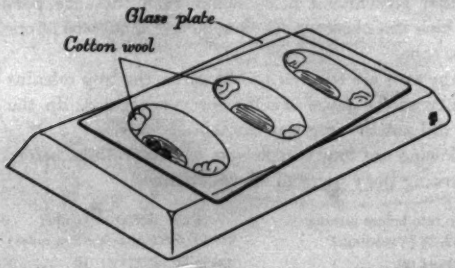

Fig. 2. Apparatus used for immersing the hearts in "small" volumes of blood or saline. Three chambers are melted out of the broad surface of a paraffin block by means of a hot spoon.

[1] Determined colorimetrically by the Dale-Evans method.

Indeed three quite different methods have been employed all of which gave the same result.

The hearts after isolation in the way described were placed in troughs hollowed out of a block of solid paraffin (Fig. 2). These troughs were roughly the shape of half an egg, about 60 mm. long × 40 broad × 8 deep. A small amount of Ringer was placed in the trough. Care was taken to have the sinus upwards lest its beat should be influenced by contact with the chamber (this precaution was found to be necessary). Its relatively large exposed surface keeps the fluid aerated. At the ends of the block were placed two pieces of wet cotton wool. A glass plate covered the whole, thus forming a moist-chamber. Care was taken that none of the Ringer from the cotton wool reached the chamber. A light can be projected through the thin bottom of the chamber and the beats of the heart or sinus easily observed with a dissecting microscope.

The following table gives some typical examples. In most cases the quantity of Ringer's solution was 0·2 c.c.

Pulse rate in intact frog after effect of pithing had disappeared	Pulse rate after removal to Ringer
18–19 H.	34
25 S.	36
18 H.	42
28–29 H.	45–47
26 S.	34
16–21 H.	34

H. Heart. S. Sinus only[1].

In order to confirm the finding that the acceleration was due to the change to Ringer's solution, the heart was placed in one cell on the paraffin block in its own blood and then transferred to an adjacent cell in Ringer's solution.

Rate of pulse in frog's blood	Maximal rate of pulse in Ringer
24 (10 identical readings over 20 minutes)	38–40 (23 readings in 27 minutes)
18–21 (40 readings over 45 minutes)	44–45 (over 12 minutes)
20–26 (5 „ 15 „)	52

Further, the hearts of two frogs were isolated and the pulse rate of each was measured in the following media in the order given: (*a*) in its own blood; (*b*) in the blood of the other; (*c*) in its own blood again; and (*d*) in Ringer's solution.

[1] The sinus was excised by cutting through the auricular wall as near as possible to the sinus, so that no lesion was made in the sinus wall but a minimal portion of auricle adhered to it.

	(a) Pulse rate in its own blood	(b) Pulse rate in the blood of the other	(c) Pulse rate in its own blood	(d) Pulse rate, maximal reading in Ringer's solution
Heart 1.	31–30 (46 readings extending over an hour)	30–32	24–28 (20 minutes)	54 (after 6 minutes)
Heart 2.	29–31 (9 readings in 20 minutes)	34–36	29–31	54 (remained high for 15 minutes)

The second method for testing the effects of removal to Ringer's solution differed from the first essentially by putting the heart (or sinus) in quantities of Ringer which were large compared to the heart itself and bubbling oxygen through the Ringer which bubbling served three purposes: (1) stirring to maintain a uniform temperature[1], (2) oxygenating, and (3) driving away the CO_2 and so maintaining the constancy of reaction of the fluid.

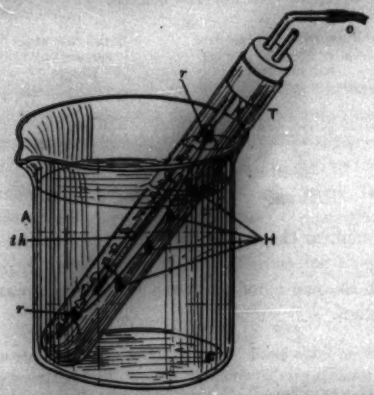

Fig. 3. Apparatus for immersing the hearts in "large" amounts of saline. A large test tube (*T*) with 50 c.c. Ringer's solution or less was immersed in a beaker with water (*A*) to keep its temperature constant. The hearts (*H*) were suspended by tying them from one of the aortæ stumps or from a little flap of pericardium to a silk thread, affixed by its two ends to the tube (*o*) conveying the oxygen by means of two small rings (*r*) made of rubber tubing. A small thermometer (*th*) was also attached along the tube.

[1] By using saline with suspended erythrocytes, observations under a magnifying glass ascertained (1) that the hearts were pumping fluid in and out, and (2) that the fluid was put in motion inside the tube by the bubbles of gas travelling along the upper slant wall.

The Ringer's solution was put in a test tube (Fig. 3) and to the tube by which the oxygen entered was attached (1) a thread on which usually four excised hearts were mounted, and (2) a small thermometer.

Pulse rate *in situ* after pithing effects had disappeared	Maximum pulse rate in Ringer's solution
22–23	44
27–28	49
33–34	40
33–34	44
22–23	45
22–23	36
18	34
21–22	35

Some experiments were carried out by the perfusion of the heart *in situ* with Ringer's solution. The fluid entered through the inferior vena cava and escaped through the aortæ.

Pulse rate before perfusion	Maximal pulse rate when perfused with Ringer's solution
18 (for 20 minutes)	34
25 („ 10 „)	42

In all these cases the maximal heart beat is reached not immediately but usually in about 5–15 minutes after treatment with Ringer's solution.

Period (ii).

From the time of maximal frequency onwards the heart rate as observed by any of the above methods gradually falls. The time taken for the complete descent varies from 15 minutes to 2 hours.

The principal cause of the variation appears to be the volume of Ringer's solution bathing the heart. If it is so very small, say 0·1–0·2 c.c., as may be used in the paraffin trough, the time necessary to produce a steady state is short. If on the other hand the volume is large as in the test tube, or virtually infinite as in the case of perfusions in which the fluid only traverses the heart once, the time is correspondingly long.

The above observation may explain the discrepancy between the statement of Colle and Noyons(2), who, perfusing the heart with a continuous stream of fluid, say that an hour and a half perfusion must elapse before the heart reaches a steady rate, and Dorothy Dale and Thacker(3), who regarded 15–20 minutes as sufficient; they used a Symes canula in which a small quantity of fluid alternately is expelled from and re-enters the heart.

Fig. 4 shows six typical tracings of the effect of immersion in Ringer's solution and its subsequent approach to the steady state in

"large" quantities of Ringer and in "small" quantities. Each set of curves covers a physiologically considerable range of hydrogen-ion

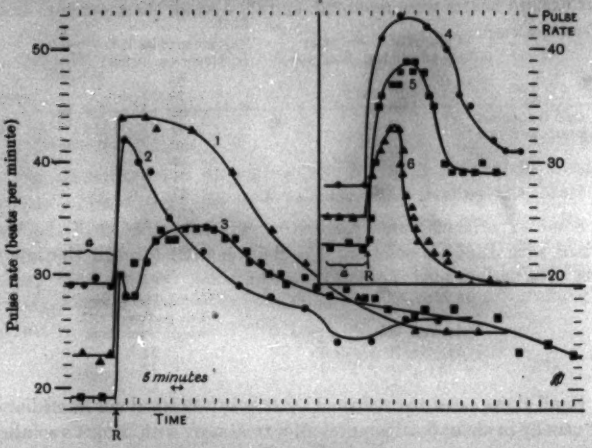

Fig. 4. Six typical tracings to show the relations between the time taken by the pulse rates to reach the steady level and the volumes of saline bathing the hearts. Heart 1 was immersed in 12 c.c. Ringer's solution; heart 2 was perfused *in situ* (saline volume virtually = ∞); heart 3 was immersed in a small amount of Ringer's solution frequently renovated, and hearts 4, 5 and 6 were immersed, respectively, in 1 c.c., 0·5 c.c. and 0·2 c.c. of bathing fluid. *R* shows the moment when the hearts were immersed in the fluid, and *a* the rates observed immediately before.

concentrations, the main phenomenon described not appearing to be connected with it.

It follows from what has been said that if a heart is kept in a small quantity of Ringer's solution and has attained a steady state, say in half an hour, and is then removed to another fresh sample of the same Ringer's solution an acceleration will occur, a fact of which sufficient account has not been taken in experiments in which the heart is moved from a solution of a given composition to that of another.

Period (iii).

Once the heart has reached a steady frequency it may be maintained so for a very long time. Fig. 5 shows 3 experiments each of which lasted for 7 hours in which the hearts maintained at a degree of constancy consistent with being regarded as steady. In any of these cases a sudden

increase or decrease of 5 beats would constitute a variation requiring some explanation.

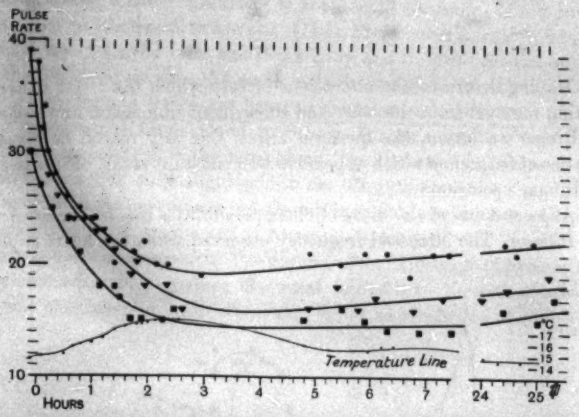

Fig. 5. Gradual fall of pulse rate after immersion in 50 c.c. Ringer's solution (*p*H 7·8) and subsequent attainment of steady state. The descent took place notwithstanding that temperature rose in the meantime about 3° C.

A number of experiments were carried out over periods of 24 hours or thereabouts. The usual result has been a slight progressive rise in the heart rate, the largest variation noted being about half a beat per hour. Thus:

Volume of fluid	Temp. ° C.	Initial *p*H	Hours after attainment of steady state	Heart beats
10 c.c.	13·8	7·6	0	16
—	13·8	—	5	18
—	13·8	—	22	29

That degree of variation was however quite exceptional. Out of 18 experiments, 14 on the heart and 3 on the sinus, the following were the differences between the initial and final rates.

Final variation in rate	+14	13	11	10	8	6	3	1	0	−4	−12
Number of experiments	1	1	1	1	2	2	2	1	3	3	1

As an example of constancy the following experiment on the sinus may be cited.

Volume of fluid	Temp. °C.	Initial pH	Hours after attainment of steady state	Rate
0·5 c.c.	15·5	7·3	0	36
—	15·5	—	1	37
—	15·0	—	17	39
—	15·0	—	26	37

Having described the alterations of rate which the heart undergoes during removal from the body and subsequent immersion and residence in Ringer's solution, the question arises, Can any reason be given for the rise of frequency which appears to be a constant result of transference to Ringer's solution?

At least some of the more obvious possibilities can be excluded.

Pithing. The increased frequency observed when the heart is placed

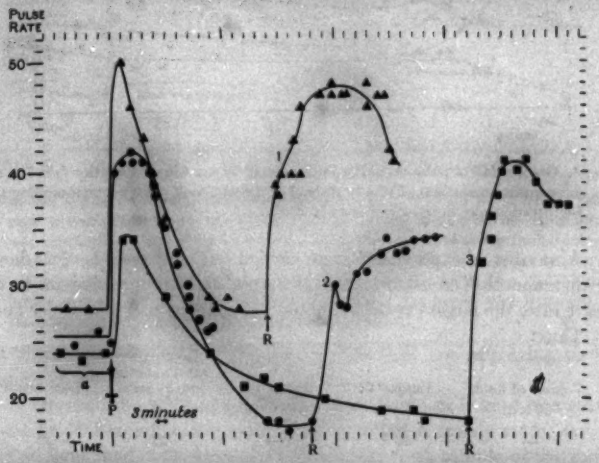

Fig. 6. Sample tracings showing that acceleration due to transference from blood to the saline medium is independent of the one brought out by the act of pithing. *P* shows the moment when pithing of the frog was performed, and *R* when each heart was removed from the body and transferred to Ringer's solution. Thus, *a* = pulse rates in the intact frogs, and *P*–*R* = rates observed in the hearts *in situ* after pithing and until the steady state was reached and maintained. 1 and 2 represent isolated sinuses in saline, and 3 corresponds to a whole heart.

in Ringer does not seem to depend on the length of time which elapses between the act of pithing and the removal of the heart. Fig. 6 shows

three hearts which were transferred at very different times after pithing. Yet in each case the acceleration was marked.

Temperature. Clearly the effect is not due to alteration of temperature as may be shown in two ways. (1) In those experiments in which the heart was removed from the frog's body and dipped into the saline fluid the temperature was the same in both situations to within about 1° C. (2) In those in which the heart or sinus was removed from about 0·1 c.c. heparinized frog's blood to 0·1 c.c. Ringer's solution this was done to an adjacent cell in the same block. The alteration in temperature can have been but very slight whilst the alteration in frequency was often over 20 beats per minute, an alteration which would have required a rise of about 10° C. in the region between 20–40° C.

Hydrogen-ion concentration. The general effect of increase of hydrogen-ion concentration is to reduce the sinus frequency. Nevertheless the particular phenomenon under discussion is not an alteration of reaction

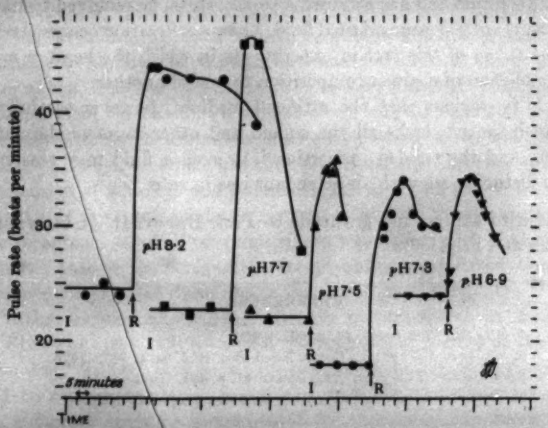

Fig. 7. Five tracings to illustrate the general effect of [H⁺]. In each tracing, after an initial period *I–R* corresponding to the rates in the intact animal, at *R* the hearts are transferred to Ringer's solution of *p*H 8·2; 7·7; 7·5; 7·3 and 6·9 respectively.

between frog's blood and Ringer for it is observable whether the sinus be transferred to Ringer (*p*H 8·2) which is more alkaline than frog's blood or Ringer's solution which is more acid (6·9).

In Fig. 7 experiments five hearts are shown, which hearts were transferred from their own blood to Ringer's solutions given above

(8·2–6·9 *p*H). The bigger accelerations corresponded to the alkaline side.

Into the effects of variations of the particular ions in the Ringer's solution I have not gone. It may however be mentioned that Zwaardemaker's(4) figures of two hearts placed in Ringer's solution poor in K show a similar acceleration.

Conclusions.

1. It has been definitely shown that when a frog's heart is removed from the body, and is immersed in Ringer's solution, the rate abruptly rises, falls gradually and finally reaches a steady level. It has been moreover shown that these changes are not related to the act of pithing or to the trauma incident to removal; they are associated with the immersion in an alien fluid.

2. If a frog's heart, after it has been kept in a small quantity of Ringer's solution and has attained a steady state, be removed to another fresh sample of the same original fluid, a new acceleration occurs. Account must be taken of this fact in experiments in which the heart is moved from a solution of a given composition to one of another.

3. It is obvious that the artificial medium, however carefully the ions are balanced, lacks all the colloid and other units of the natural medium, and the sudden immersion into such a fluid may reasonably lead to disturbances with an attendant rise in rate.

I wish to express my gratitude to Prof. Barcroft for his valuable criticism and help.

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SOME EFFECTS OF EMOTION ON THE VOLUME OF THE SPLEEN.

By JOSEPH BARCROFT.

(*From the Physiological Laboratory, Cambridge.*)

Hargis and Mann(1) in 1925 published plethysmographic tracings of the striking effects of a number of emotions on the volume of the dog's spleen. Previous papers from this laboratory record not only that the spleen contracts when exercise is taken but that it also contracts in the dog when the animal is subject to certain emotions which, when sufficiently accentuated, culminate in muscular movement of a violent character. The nature of the "motive" did not seem to be very material: it might either be benign as in the case of an animal which was all eagerness to follow its master and which ultimately insisted on doing so, or malign as in the case of an animal which attacked its master for "making much of" a companion of which it was jealous(2). It was shown also that the contraction was the result of direct nervous stimuli to the spleen in so far as in an animal, one-half of whose spleen was denervated, that half did not participate in the contraction except under great stress: the whole spleen contracted, due doubtless to the secretion of adrenaline.

Further experiments showed that the picture shown by Barcroft and Stevens ((2) p. 18) (in which the innervated portion of the spleen of the dog "Tilley" contracted on ascending the laboratory staircase) could be reproduced by mere anxiety to follow the attendant, whilst with sufficient emotion accompanied by struggling the whole spleen contracted.

The present paper deals with some points designed to expand or make more certain the phenomena described. It is somewhat disjointed owing to the difficulty of obtaining suitable preparations on which to work. This difficulty centres largely about the very great differences in temperament exhibited by different animals. Unless work is undertaken on a much larger scale than has been attempted, information of the sort desired can only be obtained when the occasion offers. These

differences of temperament are well known to all experimenters who have studied the effects on the nervous system on the viscera of animals leading an approximately normal existence; notably of course are they recorded and commented upon by Pavlov.

Indirect evidence of the emotional contraction of the spleen has been adduced by Cannon and Izquierdo(3).

The first point to which attention was directed was the length of time for which emotional contraction may be maintained. It is sometimes stated that emotional alterations in the calibre of the blood vessels are of no significance because they (the contractions) are of so transient a nature.

For this purpose an animal ("Tilley") was used which has already been described. Half of its spleen had been denervated and temperamentally the dog was extremely attached to its keeper and always anxious to follow him. The animal was laid out on the table and held lightly in position by another attendant. The keeper was sent out of the room, on which the spleen of the dog contracted, owing to anxiety to follow him. When the spleen showed any sign of relaxation the keeper was recalled, and after the dog had seen him he was again sent outside and again with consequent contraction. This procedure was kept up for half an hour, and appeared capable of being maintained indefinitely.

Fig. 1 shows the contour of the exteriorized spleen throughout this experiment, the numbers indicating the volume of the spleen relative to that during the period of rest preceding the experiment.

The contours show that the whole spleen was involved, in this the experiment was unlike others performed over short times at an earlier date; no significance as regards adrenaline secretion can be attached to this fact for the following reason. Two hundred and twenty-seven days had elapsed since the denervation of the lower half of the spleen. Sixty-five days later the animal was killed and post-mortem preparations, stained with gold chloride, showed nerve endings in all parts of the spleen. These nerve endings were much less numerous in the portion which purported to have been denervated. It seems probable that coupling (1) the alteration in the splenic response, (2) the post-mortem finding, and (3) the care with which the denervation was attempted, invasion of the denervated portion from the innervated portion was progressing.

The above experiment was repeated with unessential variations on several occasions.

With regard to the effect of adrenaline it is well known that adrenaline

does cause contraction of spleen. It has been shown, moreover, by Binet(4) and his pupils that various procedures such as asphyxia in-

Fig. 1. For explanation see text. Figures represent the approximate volumes of the spleen reckoned as a percentage of the volume at the commencement of the experiment.

volving adrenaline secretion produce an increase in the red blood count, which increase does not take place in the absence of the spleen. Feldberg has shown that in these cases actual contraction of the spleen takes place(5). Nevertheless it seemed desirable, before attributing to it any of the phenomena observed, to test the effect of adrenaline actually

Fig. 2. Effect of injection of 0·05 mg. adrenaline (intravenously) on size of spleen. Upper figures represent minutes after injection; lower figures as in Fig. 1.

Fig. 3. Effect on injection of ephedrine. Figures as in Fig. 2.

on the exteriorized spleen of the animal in question, the more so as Dr Marcou desired to test the effect of ephedrine on the organ. The results presented nothing anomalous. The operation was done under urethane anæsthesia, the adrenaline produced an intense contraction of the spleen. Its size became reduced to that of the spleen of the subsequently dead animal. The contraction passed off in a few minutes and was followed by a dilatation, which seemed to be at its maximum about 13 minutes after the injection (see Fig. 2).

The ephedrine produced a marked contraction also but not quite so intense as that of the adrenaline (which may of course have been a matter of dose): the contraction wore gradually away, but even after 50 minutes the spleen was only about half its original size, indeed it was over 2 hours before it had certainly attained to approximately its resting dimensions (see Fig. 3).

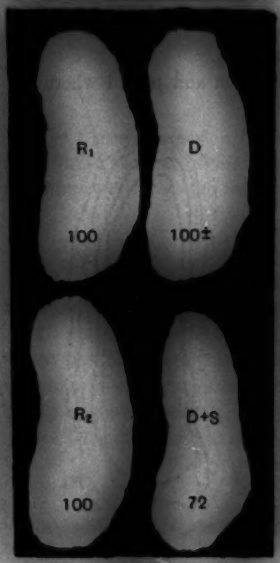

Fig. 4.

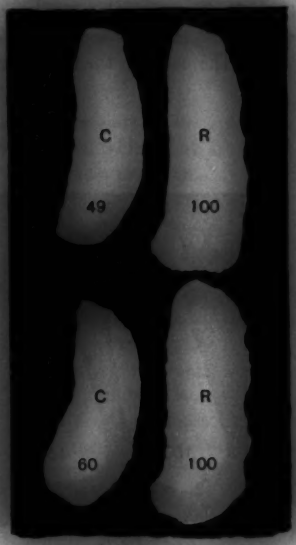

Fig. 5.

Fig. 4. R_1 and R_2 = animal at rest. D = confronted with clean duster. $D+S$ = duster which smells of cat. Figures as in Fig. 1.

Fig. 5. R = rest. C = hears cat "mew." Figures as in Fig. 1.

Passing to another phase of the subject an attempt was made to grade the emotional stimuli and to discover what grade of stimulus evoked the response (*a*) of the spleen, and (*b*) of voluntary muscle.

These experiments were carried out on a dog called "Dimple" in which the desire to pursue any cat in the vicinity was rather, but not too strongly, marked.

(*a*) If this dog were laid on the table and a clean duster dangled in front of its nose, no notice was taken of it and the size of the spleen remained unaltered. If for that duster another was substituted which had been in a basket where there was a cat, there was an appreciable though not large contraction of the spleen. There was no gross muscular response, in the sense of effort to move. It is not possible to say whether there was heightened tone of the skeletal muscles (Fig. 4).

(*b*) The cat, being in an adjacent room, if it were caused to "mew," a further but still incomplete contraction of the spleen would take place (Fig. 5). The muscular movement associated was at most pricking up of the ears, the movements of the eyes and head.

(*c*) The cat was then brought into the room and placed where the

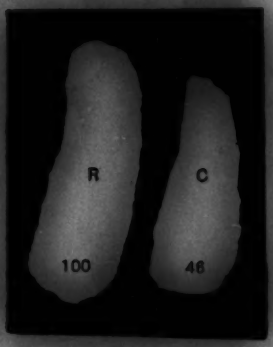

Fig. 6.

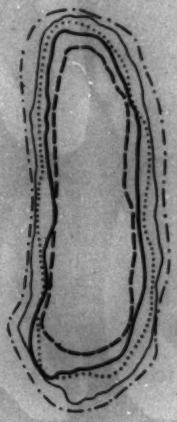

Fig. 7.

Fig. 6. *R*=rest. *C*=sees cat. Figures as in Fig. 1.

Fig. 7. —·—·— rest. —— smells cat. ..... hears cat. ——— sees cat. — — chases cat.

dog could see it, and about two feet away. This procedure was associated with a further contraction of the spleen and muscular restlessness on the part of the dog (Fig. 6).

(*d*) Finally the dog was told to chase the cat which it was allowed to do for about a quarter of a minute, with the result that its spleen contracted still more. In one case, that of June 5, the contraction was probably almost maximal, at all events the surface of the spleen (13·5 sq. cm.) approached closely to that in the animal post-mortem (13 sq. cm.). This dog was killed on 30 October following.

The following table gives a numerical statement of the effects observed: see also Fig. 7.

Approximate volume of dog's spleen expressed on a percentage of the volume at rest before the experiment.

Date	Rest	Smells cat	Hears cat	Sees cat	Chases cat
29 March	100	—	64	—	26
2 April	100	—	—	45	28
3 April	100	—	—	46	30
7 April	100	—	—	34	25
4 May	100	72	51	39	—
5 June	100	62	54	33	13

The figures suggest a better response on the part of the spleen at some times than at others. The measurements on which they are based are, however, too rough for any stress to be laid on this point.

Summary.

1. By keeping a dog in a state of mental anxiety its spleen may be maintained in the partially contracted condition for at least half an hour.

2. The known effect of adrenaline in producing contraction of the spleen has been confirmed in the exteriorized preparation, and a single injection of ephedrine has been shown to produce a contraction which passes off slowly, lasting over 60 and under 150 minutes.

3. The smell, sound, sight or pursuance of a cat produces contraction of the spleen of a dog of suitable temperament. The degree of contraction is greater in the order of stimulus given.

My thanks are due to the Government Grant Committee of the Royal Society for a grant from which part of the expense of the above research was defrayed.

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QUANTITATIVE OBSERVATIONS ON THYROXINE AND ALLIED SUBSTANCES.

II. Effects on the oxygen consumption of rats.

By J. H. GADDUM.

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It was thought desirable to amplify by other methods the results obtained with tadpoles (1) in the study of the effects of natural and synthetic thyroxine and of various intermediate and related compounds prepared by Dr C. R. Harington. For this purpose it was decided to measure their action in increasing the gaseous metabolism of mammals.

In the early investigations of the effects of thyroid preparations, an account of which will be found in Grafe's monograph (2), no attempt was made to obtain quantitative results. Thyroxine was first isolated by Kendall (3) in 1914, and was found to produce on injection all the known effects of thyroid preparations. This discovery was followed by various attempts to devise a reliable method of measuring thyroid activity. Plummer and Boothby (4) studied the effect of thyroxine on the basal metabolism of 69 cases of myxœdema and came to the conclusion that each milligramme of thyroxine would produce a maximal increase of 2·8 p.c. of the normal rate and that this maximum occurred about 8 days after the injection. The "average variation" from this figure was only 25 p.c., and the relation appears to hold for all doses between 5 and 15 mg.

Various German writers, using smaller doses, observed comparatively larger effects, after the injection of thyroxine, which developed and passed away in a couple of days (5).

For many purposes, and in particular for the study of substances with unknown properties, it is desirable to make experiments with animals other than man. Unfortunately, the work that has been done in this direction has not hitherto given such clear results as those obtained by Plummer and Boothby.

Meta Kunde (6) has published an account of a careful investigation of the effect of thyroid preparations on *dogs*. She comes to the conclusion

that it is impossible to derive any quantitative information from the study of the gaseous metabolism, even when the same dog is used again after an interval of several months.

Haffner and Komiyama(7) measured the effect of thyroid preparations administered by the mouth and subcutaneously on the CO_2 production of normal and thyroidectomized *mice*. The effect was sometimes a rise and sometimes a fall, and its magnitude did not appear to be related to the magnitude of the dose. Nevertheless, Mørch(8) found that under suitable conditions thyroid gland, administered to mice over long periods by the mouth, regularly produced a rise in CO_2 production, and that the extent of this rise bore a constant relation to the daily dose. His technique appears to be particularly well suited to the measurement of the activity of preparations of thyroid glands. It would not, however, be convenient for the study of the effects of substances like thyroxine, which show their full activity only on injection.

Various investigators have studied the effect of thyroxine on *rats*. Hildebrandt(9) measured the metabolism of the rats by a closed circuit method. He found that, after the metabolism had reached a fairly steady level, the intravenous injection of 1·3 mg. of thyroxine per kilo produced a definite rise of metabolism. Arnoldi(10) used a modification of the Zuntz-Geppert open-circuit apparatus to measure the effect of thyroxine on the metabolism of rats. The results appear to have been rather erratic. Abderhalden and Wertheimer(11) measured the effect of the subcutaneous injection of 0·3 mg. of thyroxine in increasing the CO_2 production of rats weighing about 100 g., and found that it was greatest if the rats were fed on meat, and least if they were fed on oats. A fat-diet produced an intermediate effect. Ergotamine produced a fall of metabolism. Haffner(12) found that the injection of doses of 0·5 to 5 mg. of thyroxine per kilo produced definite effects on the gaseous metabolism of rats, but that their sensitivity varied so much that they could not be used for the assay of thyroid preparations.

The fact that many observers have found it difficult to obtain consistent results serves to emphasize the importance of keeping all the conditions of the experiment as constant as possible.

General methods.

A description of the apparatus devised for the purpose of these experiments has already been published by A. N. Richards and L. W. Collison(13). I am much indebted to Prof. Richards for the opportunity of using this apparatus.

The animal is placed in a closed chamber and the carbon dioxide and water, which it produces, are absorbed by soda-lime. Oxygen bubbles in through liquid paraffin to keep the pressure constant in the chamber, and the entrance of each bubble is recorded on a drum. The oxygen consumption is determined from the rate of formation of the bubbles.

In the early stages of the work small rabbits were used. Their sensitivity to thyroxine was found to vary. It was thought that this was due to the difficulty of maintaining a rabbit's diet absolutely constant. With the object of standardizing the conditions as much as possible, rabbits were eventually abandoned in favour of rats. These were all male white rats about 1 year old, descended from Wistar stock. They had been used in this Institute for breeding and they weighed between 220 and 290 g. It is probably preferable to use males, because female rats have an œstral metabolic cycle(14), and also because Schoeller(15) found that male mice were more sensitive to thyroxine than female mice. Adult rats were used because they were more likely to be constant over a long period, and also because the response of young animals to thyroid has been stated to be abnormal(16).

The rats were kept in a room the temperature of which was constantly about 20° during the winter, but rose occasionally to 28° in the summer. The thermostat in which the metabolism of the rats was measured was kept at a temperature of 25°. They were fed on a synthetic diet composed as follows:

Rice starch	60 g.
Casein	30 g.
Marmite	5 g.
Cod-liver oil	3 c.c.
Salt mixture	7 g.
Water	105 c.c.

The salt mixture was composed as follows:

$NaCl$—21 g.; Mg_2SO_4—66 g.; $NaH_2PO_4.H_2O$—42 g.; K_2HPO_4—114 g.; Calcium acid phosphate—65 g.; Ferric citrate—24 g.; Calcium lactate—73 g.

Of this 20 g. was given to each rat daily. Any that was left was removed from the cages, in some cases 9, and in some cases 17 hours before the observations were made. The shorter interval is probably sufficient because, according to Cramer and McCall's observations(17), the excess metabolism which follows the eating of food does not last more than 8 hours. The longer interval of 17 hours was eventually adopted. The

rats were trained to eat their food in the afternoon, and a reading of their oxygen consumption was taken each morning.

Elimination of extra metabolism due to exercise.

Any movement of the animal produces a distortion of the record and irregularities in the rate of formation of the bubbles. It is thus possible to tell without difficulty which parts of the record correspond to periods during which the animal was at rest. The prolonged administration of thyroid preparations has been found by Wang(18) to depress the spontaneous activity of rats, measured in a revolving cage. Such an effect would produce a depression of the total metabolism, and interfere with the measurement of the effect of thyroid in increasing the basal metabolism. The records of a large number of experiments on rats have been examined with the object of confirming this observation. The rats are kept warm (25°) in the apparatus and on the whole they remain quiet, but they are seldom still for more than 15 min. at a time. During the course of an experiment they invariably devote a portion of the time to cleaning themselves, or to other forms of exercise. The total length of the time during which they remained at complete rest on different days before and after the administration of thyroid was measured, and it was found that, as in the case of Wang's experiments, large variations occurred in the activity of the rats. The comparatively small injections of thyroid used in these experiments were generally followed by a fall in the activity of the rats, but occasionally a rise was seen. In any case it is clearly desirable to eliminate the effects of irregularities in the activity of the rats. The way in which this was achieved is described below.

Fig. 1 shows the oxygen consumption of a rat during a period of 3 hours in the apparatus. The periods during which the record showed that the rat was moving about are marked at the bottom of the diagram. It will be seen that the oxygen consumption is notably increased during each such period and for some time after. Fig. 1 also shows a large rise in oxygen consumption and in activity towards the end of the experiment. Such rises were usually seen as soon as the rat had become accustomed to the termination of the experiment at the end of 3 hours. It is thus probably desirable in experiments on the metabolism of rats to make a practice of leaving the rat in the apparatus for some time after the experiment is finished, in order to eliminate this period of restlessness. Such a precaution was, of course, unnecessary in the experiments here described, since periods of activity were automatically excluded. It was,

however, necessary to exclude the period at the beginning of the experiment before the temperature of the chambers had reached a steady rate. For this reason the record of the first hour after the rats were placed in the chambers has invariably been neglected.

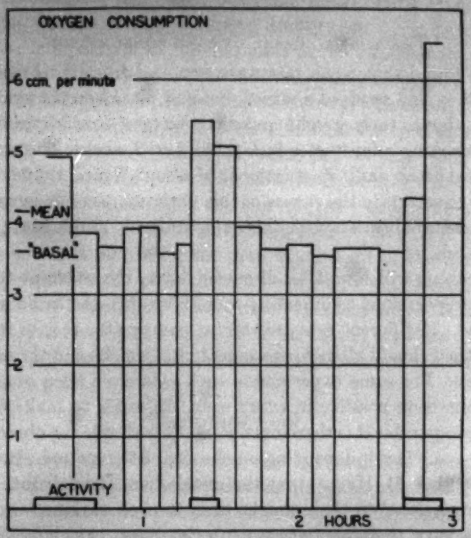


Fig. 1. Oxygen consumption of normal rat weighing 227 g. during the course of a single experiment. The "basal" oxygen consumption, calculated in accordance with the convention given in the text, is marked on the left of the diagram. The periods during which the record showed that the rat was moving are marked at the bottom.

In order to obtain a measure of the basal metabolism the rats were placed in the chambers for 3 hours each morning and the bubbles, etc., were counted over intervals of from 5 to 15 min. in all the periods during which the animal was at rest, but neglecting the few minutes immediately following active movement, when the rate of formation of the bubbles was obviously much increased. The total period so measured varied from 25 to 75 min. in different experiments. This includes, of course, periods during which the oxygen consumption was still slightly increased after exercise. The convention was adopted of selecting the lowest of these readings, so as to make up a total period of between

20 and 30 min. The value of the "basal" oxygen consumption, calculated according to this convention, is shown in Fig. 1. The mean value for the whole 3 hours is also shown in this figure. It was found that the mean oxygen consumption was usually 15 to 25 p.c. greater than the "basal" oxygen consumption.

The normal basal oxygen consumption.

It was found that when rats were removed from the breeding cages in which they had received a mixed diet and placed on the synthetic diet described above, their weight and their oxygen consumption both fell slightly, reaching a fairly steady level in 2 or 3 weeks. The oxygen consumption showed daily fluctuations, of about 5 p.c., round this level. Attempts to ascertain the causes of this variation were unsuccessful, and when the metabolism reached this comparatively stable state the injections were made.

For reasons which will be discussed later, the attempt to compare different preparations by injecting them both into the same animal was abandoned. If different rats are to give comparable results it is clearly desirable they should all have the same initial weight and the same initial metabolism. For some experiments such rats have been obtained, but this has not been possible in every case. In order to make the results properly comparable it is therefore necessary to divide the observed metabolism by some factor depending on the size of the animal. It was found by A. V. and A. M. Hill(9) that the metabolism of adult rats was more nearly proportional to their weight than to their surface. Oxygen consumptions have therefore, where necessary, been calculated in c.c. per kilo per min. The weights of the rats used in the experiments here described did not vary sufficiently to enable evidence either for or against Hill's finding to be obtained.

The mean of 66 observations on 22 rats, taken after the metabolism had reached a steady value, was $14{\cdot}44 \pm 0{\cdot}07$ c.c. (corrected to 0° and 760 mm.) per kilo per min. The figure 0·07 is an estimate of the probable error of this determination of the mean. It is deduced from the standard deviation for a single observation in the series, which was 0·85 c.c. per kilo per min. or 6 p.c. The mean of the readings obtained in the winter was about 7 p.c. higher than that of those obtained in the summer. This is probably to be attributed to the incompletely controlled variation in the temperature of the room, where the animals were kept when they were not in the apparatus. The variation of the metabolism at any one time was, therefore, probably less than that indicated by the estimate

of the standard deviation given above. The mean oxygen consumption is considerably lower than the normal values obtained by Hildebrandt and Haffner. The difference is probably to be attributed to differences in diet, and in the temperature at which the measurements were made, as well as to the fact that these observers measured the total oxygen consumption, instead of that occurring during rest.

From the composition of the diet used in the experiments described above, it may be calculated that the mean oxygen consumption is equivalent to about 700 large calories per square metre per 24 hours. This figure is lower than Rubner's Law predicts (1000 calories per square metre per 24 hours), as was to be expected, since the readings were taken at 25° instead of 16°, and since the animals were at complete rest while the measurements were taken. They are more directly comparable with the results obtained by Goto(20) with fasting rats at 25°. His figure was 834, which is about 20 p.c. higher than that found in the experiments described here. Goto's observations were made on the total metabolism over 7 or 8 hours, and, as has already been stated, the total oxygen consumption has been found to be 15 to 25 p.c. higher than the "basal oxygen consumption," so that the agreement between the two sets of observations is good.

Thyroidectomy.

It was expected that the removal of the thyroid glands would stabilize the oxygen consumption of the rats during the preliminary control period and render them more susceptible to the influence of thyroxine, and thus more suitable for use in tests. It will be seen later that not one of these expectations was realized.

The technique of the operation, which was first described by Cristiani(21), is not difficult to acquire, but care must be taken to avoid injuring the recurrent laryngeal nerve which is closely applied to the inner surface of the lobes. Glands have been removed from about 12 animals under ether, and in one or two cases their identity has been confirmed by microscopical examination. The rat has only two parathyroids, and these are both inevitably removed with the thyroid(22). The rats have usually developed tetany after the operation, but they were given milk, and except in one or two cases the symptoms disappeared within 24 hours under this treatment and did not recur when the rats returned to their synthetic diet. The absence of thyroid tissue has been confirmed post-mortem, in most cases by microscopical examination of sections of tissues in the region from which the gland had been removed.

After the operation the metabolism usually fell to below the normal range. In one case in which the fall was followed it occupied 5 days. The experiments on which conclusions as to effects of thyroidectomy are based were all carried out on rats whose metabolism was less than the normal value given above by more than twice the standard deviation for a single observation, *i.e.* by more than 12 p.c. It is probable that in these rats the removal of the gland had been effective in abolishing its function. The small unexplained daily fluctuations which occurred in the oxygen consumption of these rats were no smaller than those seen in normal rats, which for this, and other reasons, have been used in most of the experiments to be described.

One of the thyroidectomized rats, which was kept for 9 months after the operation, became fat and sluggish in its behaviour. It was then given 5 grains of dried thyroid daily for 9 days, as a result of which its oxygen consumption rose to a little over double its previous value, its weight fell by 18 p.c. and it became much less placid in appearance. It did not develop exophthalmos as might have been expected from results of the experiments of Meta Kunde with rabbits(6).

Method of administration. Controls.

Except where otherwise stated the preparations have been dissolved in $N/100$ sodium hydrate (2 mg. per c.c.), and injected subcutaneously at about 5.0 p.m. The first observation has been taken between 11 a.m. and 1 p.m. the next day. The observations have usually been confined to the effects of a single injection.

Many of the substances tested are extremely insoluble, and in some cases the appropriate dose has been injected dissolved in as much as 8 c.c. of $N/100$ NaOH. The injection of such a quantity of NaOH without the addition of any other substance, sometimes, but not invariably, produces a slight rise in the oxygen consumption, as does that of sterile distilled water. Such rises do not exceed 20 p.c. of the original oxygen consumption, and they reach a maximum in the first daily reading and then rapidly disappear. Adequate doses of thyroxine and allied substances are found to cause much larger and more prolonged rises, which are easily distinguished from these unspecific effects. In two experiments in which 10 c.c. of $N/50$ NaOH and 10 c.c. of $N/50$ HCl were injected daily for 4 days, no effect was obtained comparable with the specific effect.

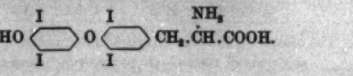
Two experiments were carried out in which 4 mg. per kilo of racemic

thyroxine were injected subcutaneously in watery suspension, and intravenously in solution. In the former case no effect at all was obtained, and in the latter case the rise which occurred was only about 10 p.c. of the original oxygen consumption and had disappeared on the third day. It was thus scarcely distinguishable from the unspecific effects produced by the subcutaneous injection of distilled water. When this quantity of thyroxine in solution was injected subcutaneously the true specific effect was invariably clearly shown.

Results.

I am indebted to Dr C. R. Harington for most of the preparations that have been tested. A reference to a description of the method by which each of these was prepared is given with the name of each substance. The volume of oxygen consumed has in every case been corrected to 0° and 760 mm.

Racemic synthetic thyroxine (23).



Compared with man, rats in common with other small animals are very insensitive to the action of thyroxine when the dose is calculated in mg. per kilo. The effect appears more rapidly and does not last so long. When an adequate dose has been injected subcutaneously the oxygen consumption shows a definite increase after 18 hours and usually reaches a maximum in the second daily reading. With a dose of 1 mg. per kg. the fall to normal is then fairly rapid. After larger doses the fall is slower and the oxygen consumption may even continue to rise after the second reading and not return to normal till 3 weeks after the injection. The fall in weight, which only occurs with large doses, is rather slower in onset and reaches a maximum at about the time when the oxygen consumption returns to its normal value. When a large effect has been obtained, the big loss of weight is usually associated with a fall of the oxygen consumption below its original value. Some slight variation has occurred in the sensitivity of the rats, which appeared to be high in the winters and springs of 1928 and 1929, and low in the autumns of the same years, but it is not known whether this variation occurs regularly with the seasons.

Some typical results are shown in Fig. 2. These particular experiments were all carried out at about the same time, but the rats differed from

one another in weight. In order to compare the effects with one another, the oxygen consumption has in each case been divided by the weight of the rat. The reasons for this procedure in comparing the oxygen consumption of different rats have been discussed above. When a comparison is made between the oxygen consumption of any one animal

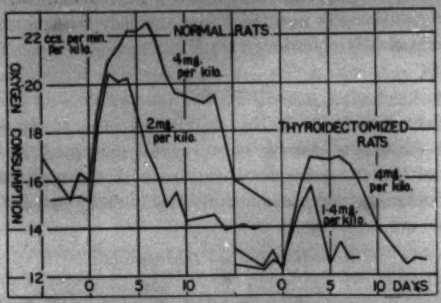


Fig. 2. The effects on the basal oxygen consumption per kilo of injecting solutions of racemic thyroxine subcutaneously.

before and after treatment with thyroid the effect on the oxygen consumption per kilo is usually larger than that on the total oxygen consumption, since the animal loses weight. Loss of weight, due to such causes as starvation, is itself associated with a proportional fall in metabolism, so that, if the total metabolism is plotted, the real increase in metabolism produced by thyroid may be obscured by secondary effects due to loss of weight. For these, and other reasons, Mørch(8) measured the activity of thyroid preparations by their effect on the CO_2 production *per gram* of mouse (except in the case of such growing mice as actually gained weight during the experiment). A similar convention has been followed in Fig. 2. This method of calculating the results was found to eliminate the fall of oxygen consumption which, as has been stated, occurs after the main effect of a large dose has passed away.

It was found that the effect of a second injection of the same dose, even when given 10 days after the oxygen consumption had returned to normal, was considerably greater than that of the first injection. A similar increase in sensitivity was observed in a small rabbit, and also in two thyroidectomized rats. This particular phenomenon did not therefore appear to be dependent on the presence of the thyroid. A similar observation was made by Boothby, Sandiford, Sandiford and

Slosse[24]. In view of these results it was decided not to attempt to compare the effects of different substances on the same rat.

The available evidence[24,25,26,27] makes it probable that the response of myxœdematous patients to thyroid administration is greater than that of normal individuals, and it was therefore rather surprising to find that the contrary appeared to be true of rats. Six injections of racemic thyroxine were made in thyroidectomized rats, and in each case the effect on the oxygen consumption was smaller than that produced by the same dose in normal rats. In one case injections were made in the same rat before and after thyroidectomy, and the second response was smaller than the first. However, though thyroxine was thus found to produce a greater absolute increase in the oxygen consumption of normal than of thyroidectomized rats, the percentage increase was about the same in the two cases.

d-Thyroxine ($[\alpha]^{21°}_{5461} + 3{\cdot}0°$) *and l-Thyroxine* ($[\alpha]^{21°}_{5461} - 3{\cdot}09$[23]).

Injections of different doses of these preparations were made at the same time in four rats of almost identical weights, and consuming oxygen

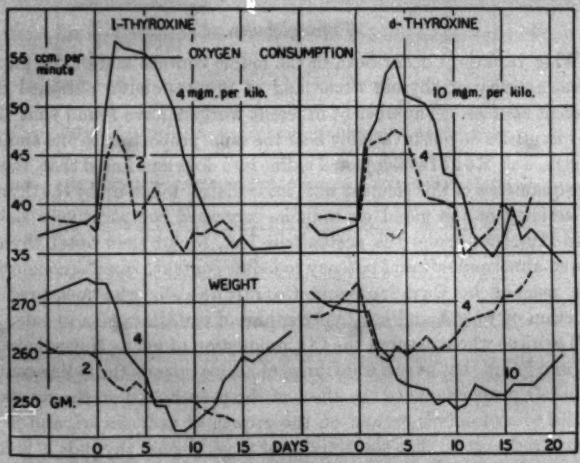


Fig. 3. A comparison of the effects of subcutaneous injections of preparations of the optical isomers of thyroxine on four rats. *l*-thyroxine had more effect than *d*-thyroxine both on the weight and on the oxygen consumption.

at almost identical rates. The results are shown in Fig. 3. It will be seen that 4 mg. per kilo of *l*-thyroxine produced rather more effect, both on the oxygen consumption and on the weight, than did 10 mg. per kilo of *d*-thyroxine. On the other hand, 2 mg. per kilo of *l*-thyroxine produced rather less effect than 4 mg. per kilo of *d*-thyroxine. The latter two doses were also injected into another two rats and in both cases the injections of 4 mg. per kilo of *d*-thyroxine produced slightly more effect than either of those of 2 mg. per kilo of *l*-thyroxine. There can be no doubt that the *l*-thyroxine was more potent than the *d*-thyroxine, since the effect of 4 mg. per kilo of *l*-thyroxine was much larger than has ever been obtained with the same dose either of *d*-thyroxine (two experiments) or of racemic thyroxine (four experiments); but the exact numerical proportion between the potencies of the isomers appeared to vary between about 1·5 and 3, according to the dose used.

Similar results were obtained in experiments with tadpoles, carried out by the method previously described (1). In four different experiments *l*-thyroxine has always produced more effect than the same concentration of *d*-thyroxine, but the exact ratio of their potencies has appeared to vary between 1·2 and 2.

Thyreoglobulin.

Wide variations occur both in the iodine content and in the physiological activity of thyroid tissue and of thyreoglobulin obtained from different sources. A number of different workers have found that these two quantities do not invariably bear the same proportion to one another. Marine and Rogoff (29) injected iodine into dogs and found that, though large quantities of this element were immediately taken up by the thyroid, the activity of the gland on tadpoles increased comparatively slowly. Reid Hunt (30), using the acetonitrile test, found that fœtal thyroids showed abnormal ratios of potency to iodine content. Similar conclusions were reached by Cameron and Carmichael (31) who measured the reduction of weight and the hypertrophy of certain organs in rats, and by Mørch (8) who measured the CO_2 production of mice. Nevertheless, it has been found that over a wide range of normal glands the iodine content is directly proportional to the effect of the preparation on the resistance of mice to acetonitrile (30) and on the growth of tadpoles (32), and it has been stated (30,33,34) that the activity of these normal thyroids is greater than that of the thyroxine which they contain. The inference has been drawn that thyroxine is combined in these preparations with other chemical groups which enhance its activity. The following experiments

were carried out with the object of amplifying the evidence on this important point.

A preparation of thyreoglobulin was made by Dr Harington by isoelectric precipitation at *p*H 5, and well washed with alcohol and ether. It was found to contain 0·76 p.c. of iodine. A dose of this substance and one of racemic thyroxine each of which contained 2·6 mg. of iodine per kilo of rat was mixed each day with a small portion of food, and eaten by two rats. The effects on the oxygen consumption are shown in Fig. 4.

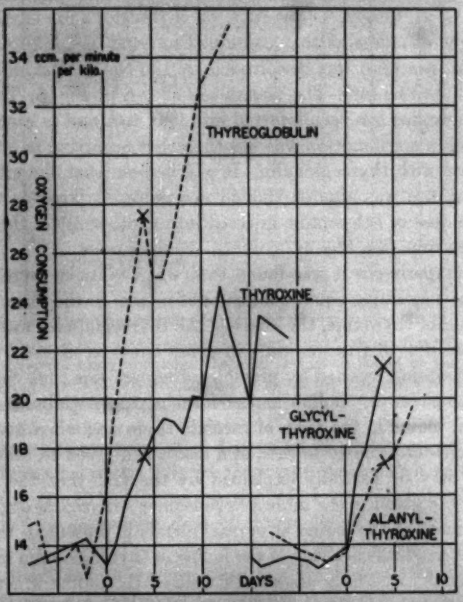


Fig. 4. A comparison of the effects of administering by the mouth a daily dose of 2·6 mg. per kilo of iodine combined in different forms. The oxygen consumption after 4 days' administration is marked in each case by a cross.

It is clear that the effect of the thyreoglobulin was considerably larger than that of the thyroxine. This difference was no doubt partly due to the fact that thyroxine is either imperfectly absorbed or destroyed in the intestine. Thus the effect of this substance, observed after several daily

doses in the food, was less than that produced by a single subcutaneous injection of the same dose (4 mg. per kilo, cf. Fig. 2). The comparative inactivity of thyroxine when given by the mouth has been observed before (6, 26, 35). Nevertheless, in the comparison of thyroxine with more complex derivatives, the substances have usually been given by this route. Reid Hunt (30), it is true, carried out an experiment in which thyroxine was injected subcutaneously, but the result cannot be said to have been conclusive. It seemed desirable, therefore, to compare the activity of the two preparations when both were given by subcutaneous injection. Accordingly, a quantity of thyreoglobulin, containing the same weight of iodine as that contained in 4 mg. per kilo of thyroxine (i.e. 2·6 mg. per kilo) was dissolved in $N/100$ NaOH and injected subcutaneously in two rats. The results are shown in Fig. 5. This dose of racemic thyroxine has been injected into four rats, and in each the effect on the oxygen consumption was less than that occurring in either of the experiments with thyreoglobulin. It will be seen that the effect of this latter substance was almost, though not quite, as large as that of an equivalent dose of *l*-thyroxine injected into another rat of the same size at the same time (see Fig. 3).

In one experiment it was found that when tadpoles were immersed in solutions containing equal quantities of iodine, in the form of thyreoglobulin and of thyroxine, the effect of the thyreoglobulin was definitely greater than that of *d*-thyroxine, and about equal to that of *l*-thyroxine.

The conclusion reached by previous observers was thus confirmed in three different ways. Iodine in the form of thyreoglobulin was more active than iodine in the form of racemic thyroxine when given by the mouth or subcutaneously to rats, and also when given to tadpoles. The difference may be partially explained by the fact that the thyroxine present in the gland is the more active isomer *l*-thyroxine (36), but since 50 p.c. or more of the iodine in normal glands is present in the form of diiodotyrosine (37) the activity of the iodine in thyreoglobulin might have been expected to be only about half that of the iodine in *l*-thyroxine. Therefore, since equal doses of iodine in these two forms were found to produce about equivalent effects, the effectiveness of thyroxine *did* appear to be slightly increased by its combination with other chemical groups in thyreoglobulin. It should, however, be understood that, as in most other cases where the activities of two substances are compared, the observed difference may possibly be due to differences in the rate at which the two substances are absorbed and excreted.

Dipeptides containing thyroxine (38).

One of the difficulties in the clinical use of thyroxine lies in the fact, mentioned above, that it has, when given by the mouth, very little effect in comparison with that of dried thyroid gland or of thyreoglobulin. Preparations of glycyl thyroxine and alanyl thyroxine were made in order to explore the possibility that they might be more effective than thyroxine when thus administered. It will be seen from Fig. 4 that

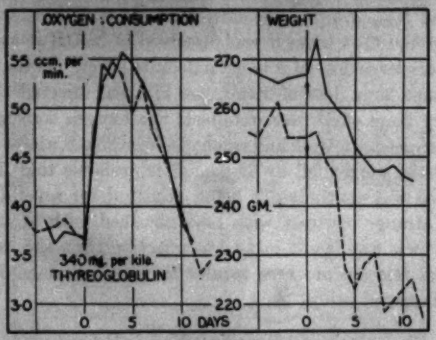


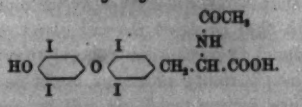
Fig. 5. The effect, on the oxygen consumption and weight, of injecting subcutaneously a dose of thyreoglobulin containing the same weight of iodine as 4 mg. per kilo of thyroxine. These results were obtained at about the same time as those shown in Fig. 3 and are directly comparable with them.

alanyl thyroxine produced after 4 days an effect almost identical with that of thyroxine itself. Glycyl thyroxine produced a rather greater effect in the same time, but even this effect was very small compared with that of an equivalent dose of thyreoglobulin. After 4 or 5 days' administration of the dipeptides, each of these rats began to eat much less of its food, and its oxygen consumption showed a sudden fall. The experiment was not continued beyond this point. These results were not considered sufficiently encouraging to warrant the preparation of more complex polypeptides.

Glycyl thyroxine was compared with thyroxine by subcutaneous injection in solution. Equivalent doses were found to produce about the same increase of oxygen consumption, but the effect of the dipeptide disappeared more rapidly than did that of thyroxine. In one experiment the very large dose of 50 mg. per kilo was injected. This produced in

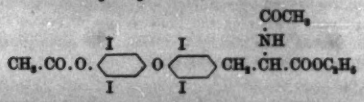
5 days a 100 p.c. rise in oxygen consumption which also then fell rapidly to normal again.

N-acetyl thyroxine (39).



This substance is identical with glycyl thyroxine except for the absence of an NH_2 group in the side-chain. It was therefore somewhat surprising to find that when it was dissolved in NaOH it was necessary to inject a very large dose of it to get a definite effect. On the other hand, when this same large dose of 50 mg. per kilo was injected suspended in water a very large effect was obtained; the oxygen consumption continued to increase for a week and reached a level 85 p.c. above its previous value, while the weight fell by 24 p.c. It is probable that, in this case, the substance was continuously being absorbed for several days. This result is in strange contrast with that obtained with a suspension of thyroxine which was found to be less effective than the same dose in solution. The relation of these results to those obtained by previous workers is discussed below.

Diacetyl thyroxine, Kendall (3).



The chemical properties of this substance, prepared according to the method given by Kendall (3), have been examined by Ashley and Harington who assign to it the above formula (39). It was found by Kendall (40) that this substance produced effects on metamorphosis, but not on metabolism. These results were confirmed by Swingle (26), who found that it produced metamorphosis in hypophysectomized and thyroidectomized tadpoles, but had no effect on the pulse rate or weight of normal men on intravenous injection. Hoskins (41) found that this substance accelerated the development of young rats on subcutaneous injection. These results have been thought to show that the effect of thyroxine on metamorphosis was due to a part of the molecule different from that affecting metabolism.

This diacetyl thyroxine is extremely insoluble in water, and it was not found possible to obtain a solution of sufficient strength for injection

without subjecting it to processes which might alter its structure. (The procedure followed by Hoskins would be likely to convert this substance into the N-acetyl derivative, the effects of which are described above.) Accordingly, 50 mg. per kilo were suspended in water and injected subcutaneously. The oxygen consumption of this rat rose, as may be seen in Fig. 6, by over 50 p.c. in 7 days.

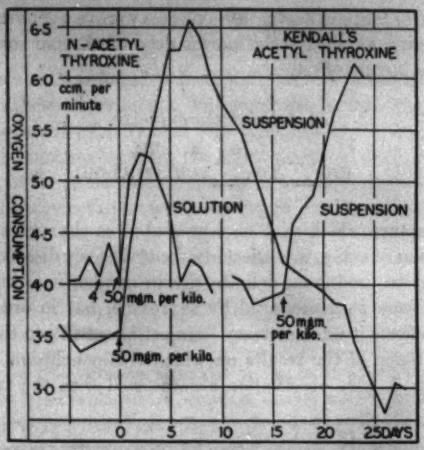


Fig. 6. Effect on basal oxygen consumption of three rats of injecting two different preparations of acetyl thyroxine subcutaneously in suspension—and in one case in solution.

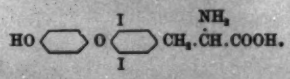
It is thus clear that both of these two forms of acetylated thyroxine may have marked effects on the metabolism. It is thought that the difference between this conclusion and that of other workers is to be attributed to the difference in the mode of administration. In the experiments described here, the active material was probably slowly absorbed over a long period, and it is known that a dose of thyroid material given slowly is more effective than the same dose given all at once.

It is, of course, possible that these acetyl thyroxines are inactive in themselves, and that thyroxine is slowly liberated from the very low concentration of these substances which was maintained for a long period in the subcutis of the rat round the site of the injection. Nevertheless, it is clearly unsafe to draw the conclusions that have been drawn from

the observation that this substance produced effects on metamorphosis, but not on metabolism, unless the substance is administered in the same way in both cases. In particular, the observation of Hoskins, that acetyl thyroxine accelerated the development of young rats, becomes less paradoxical when it is known that it probably also increased their metabolism.

It is possible that the subcutaneous injection of suspensions of one or other of these forms of acetyl thyroxine may prove a convenient method of administering an accurately known dose of thyroid material which would have a lasting effect.

3, 5-*Diiodothyronine* (23).



This substance which may be represented as thyroxine deprived of two of its atoms of iodine was injected subcutaneously dissolved in NaOH. It was found to produce a definite rise in oxygen consumption indistinguishable from that produced by thyroxine, but in order to obtain comparable effects it was necessary to inject about fifteen times as much by weight. Some of the results on which this conclusion is based are shown in Fig. 7. This observation accords well with the fact that this

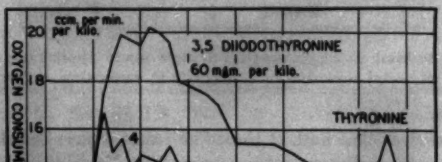
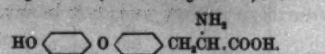


Fig. 7. Effect on oxygen consumption per kilo of three rats of injecting subcutaneously solutions of two substances similar to thyroxine in constitution, but containing less iodine.

substance was found to produce the specific thyroid effect on tadpoles (1) in concentrations considerably greater than were necessary in the case of thyroxine. The ratio of the activities of the two substances on tadpoles was found to be 40 : 1 in one experiment and rather less in others. The

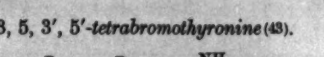
exact proportion probably depends on the conditions of the experiment, and the dose used. It was thought that 3, 5-diiodothyronine might owe its activity to its being converted by the thyroid gland into thyroxine. This possibility was excluded by the observation that it produced as large an effect on a thyroidectomized rat as on a normal rat injected with the same dose.

Thyronine (*Desiodothyroxine*) (42).



This substance was found to be entirely devoid of the specific thyroid effect seen in these experiments. The small rise in the oxygen consumption which occurred (Fig. 7) after two injections of 200 and 100 mg. per kilo was indistinguishable from the effect produced later in this rat by the injection of the same volume of soda as had been used to dissolve the thyronine. This substance was also found to be without effect on tadpoles (1).

3, 5, 3′, 5′-*tetrabromothyronine* (43).



A sample of this substance, in the molecule of which the four atoms of iodine have been replaced by bromine, was obtained through the kindness of Dr M. Guggenheim of Basel. It was found to produce a weak effect like that of thyroxine on the oxygen consumption of the rats. The results of the subcutaneous injection of solutions of this substance are shown in Fig. 8. A single injection of 10 mg. per kilo gave only the unspecific effect which may be given by any injection, but after 50 mg. per kilo the effect was more prolonged (*A*). A series of injections of 20 mg. per kilo was accordingly given to another rat, and a rise of well over 50 p.c. in the oxygen consumption was obtained, together with a fall in weight (*B*). It was thought that this effect might be due to the conversion of this bromine derivative into thyroxine in the thyroid gland. A similar series of injections was therefore given to a thyroidectomized rat (*C*). A definite but smaller effect was obtained, and the conclusion was drawn that if any chemical change such as had been postulated was responsible for all of the observed effect, the thyroid gland was not the exclusive site of its occurrence.

Similar results with diiodothyronine, thyronine and tetrabromothyronine have been reported by Abderhalden and Wertheimer (44) who

have investigated the physiological properties of a most interesting series of derivatives of thyronine.

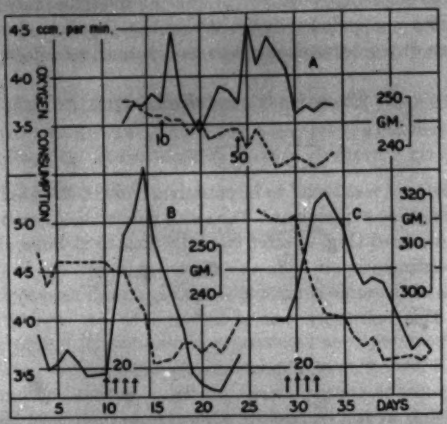


Fig. 8. Effect on oxygen consumption (continuous lines) and weight (broken lines) of injecting subcutaneously solutions of tetrabromothyronine. Injections marked by arrows. Doses in mg. per kilo. *A* and *B* were normal rats; *C* was a thyroidectomized rat.

The following three substances were found to be without definite effect on the oxygen consumption.

Substance	Dose used
Thyroxamine (thyroxine minus CO_2) (36)	25 mg. per kilo in suspension, the injection being repeated 2 days later.
$\beta\beta$-di (3, 5-diiodo-4-hydroxyphenyl) alanine (this is an isomer of thyroxine) (45)	50 mg. per kilo.
3, 5-diiodotyrosine	50 mg. per kilo daily for 4 days.

Some experiments, which were performed with thyroxamine and tadpoles (1), seemed to indicate that it had a definite effect similar to that of thyroxine, but it was found that the alcohol and acid which had been used to obtain a solution themselves had effects, and the question of the physiological activity of thyroxamine was left undecided. Since that time it has been found that small doses induce metamorphosis in axolotls (44). A small effect on rats was also obtained by the same observers. The reason for the disagreement between this observation and the experiment recorded above is not known. It is possible that in this

experiment the mode of administration was unsuitable. In another experiment a dose of 0·6 mg. in dilute HCl was injected intravenously in a spinal cat weighing 2·8 kilos. This had no definite effect on the blood-pressure such as might have been expected from its chemical constitution.

The second substance shown in the list was also found to be without effect on tadpoles(1). The result obtained with 3, 5-diiodotyrosine confirms the observations of Strause and Voegtlin(46), and of Hoffmann(47) and others.

Summary.

1. Adult male rats from standard Wistar stock were fed on a standard synthetic diet, and a record was taken of their oxygen consumption and movements during 3 hours every morning by means of the apparatus described by Richards and Collison.

2. From these records the resting oxygen consumption of the rats was determined. The normal value at 25° was 14·44 ± 0·07 c.c. per kilo per minute, or 700 cals. per square metre per 24 hours. The effects on this of the administration of thyroxine and allied substances were studied. The conclusions already reached as a result of experiments with tadpoles were confirmed.

3. The most effective way of giving thyroxine to rats is the subcutaneous injection of an alkaline-watery solution; the intravenous injection of such a solution, or the subcutaneous injection of a neutral suspension being relatively ineffective. The response to thyroxine was found to be diminished after thyroidectomy, and to be increased if another injection had been given previously.

4. When used in the pure state the naturally occurring isomer, *l*-thyroxine, had more effect, on rats and on tadpoles, than *d*-thyroxine.

5. Given orally to rats, iodine in the form of thyreoglobulin had much more effect than in the form of thyroxine. Tested on tadpoles or by subcutaneous injection in rats, a dose of *l*-thyroxine was equivalent to a dose of thyreoglobulin with the same total iodine content.

6. Two dipeptides containing thyroxine had, in equivalent doses, effects not very different from those of thyroxine itself, both when given by the mouth and when given subcutaneously.

7. N-acetylthyroxine and diacetylthyroxine (Kendall) injected subcutaneously suspended in water produced large and prolonged increases in oxygen consumption.

8. Diiodothyronine injected in comparatively large doses produced

an increase in the oxygen consumption both of normal and of thyroidectomized rats. Thyronine had no effect.

9. The substance in which all the iodine of thyroxine is replaced by bromine had a slight effect like that of thyroxine both on normal and on thyroidectomized rats.

10. Thyroxamine, diiodotyrosine and ββ-di-(3, 5-diiodo-4-hydroxyphenyl) alanine (which is isomeric with thyroxine) were without effect on the oxygen consumption.

I wish to thank my assistant, F. F. McDonald, who has carried out a very large number of rather laborious experiments.

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THE OXYGEN CONSUMPTION OF THE AURICLES OF THE FROG AND OF THE TORTOISE.

By A. J. CLARK and A. C. WHITE.

(*From the Department of Pharmacology, University of Edinburgh.*)

The authors have previously reported(1,2) the results of experiments on the oxygen consumption of the isolated heart and ventricle of the frog. This paper describes a series of similar experiments upon the isolated auricles of the frog and of the tortoise. The special advantage of these preparations is that the walls of the auricles are so thin that a full respiratory exchange can occur through the external wall, and therefore the oxygen supply of the tissue does not depend on the movements of the fluid that it contains. Hence these preparations are particularly well adapted for measuring the oxygen consumption under conditions which interfere with the emptying of the auricle, *e.g.* isochoric[1] conditions, or paralysis by drugs.

Methods.

The frog's ventricle was cut across about a millimetre from the auriculo-ventricular junction. A cannula was introduced into the auricles through the A.-V. orifice and tied in firmly. The sinus was ligatured at its junction with the great veins and the auricles removed. As much as possible of the ventricular tissue was then removed, but a considerable portion of the bulbus arteriosus was left attached to the auricle.

The tortoise's auricle was prepared in exactly the same manner except

[1] Weizsäcker(3) pointed out that the terms isotonic and isometric do not express accurately the conditions that obtain when a hollow muscular organ containing fluid is stimulated. If the organ be allowed to empty itself at a constant pressure, nevertheless the tension on the walls varies during contraction and hence the conditions are isobaric but are not isotonic. In our experiments the pressure rose slightly during contraction and hence the conditions were auxobaric. On the other hand, since the relaxed auricle is not a true sphere, hence the length of its fibres can decrease on stimulation even though the volume does not alter. The conditions are in this case isochoric and not isometric. For the sake of accuracy we have therefore adopted Weizsäcker's terminology of isobaric, auxobaric, and isochoric. We consider that it is preferable to use this somewhat unfamiliar terminology rather than to use the familiar terminology in an inaccurate sense.

that in this case it was necessary to incise the interauricular septum for about 0·5 cm. before introducing the cannula. Otherwise this septum prevented the emptying of one auricle. The tortoise's auricle could be isolated free from any ventricular or bulbar tissue.

The Ringer's fluid used had the percentage composition of NaCl 0·65, $CaCl_2$ 0·024, KCl 0·015 and sodium phosphate 0·02 ($pH = 7·6$). It was found that the auricles, when they contained Ringer's fluid alone, began to leak after a few hours, but that this was prevented by the addition of an equal volume of serum. Defibrinated and hirudinized blood also were tried, but these did not appear to possess any advantage over serum. A mixture of equal parts of frog's serum and Ringer's fluid was therefore used as the standard fluid for filling the frog's auricle. In the case of the tortoise either undiluted serum, or two parts of serum to one part of Ringer's fluid were used.

The oxygen consumption was measured in Barcroft manometers with special bulbs. The shape of these bulbs was found to be of great importance. The bulbs were made as small as possible, and a consumption of 1 c.mm. oxygen caused a movement of the manometer fluid of from 0·2 to 0·6 mm. The time that elapsed before reliable readings of oxygen consumption could be obtained was found to depend upon the position of the caustic soda receptacle. Experiments that will be described later showed that the whole of the measurable respiratory exchange took place through the walls of the auricle, and that little or none took place through the surface of the fluid in the cannula. The carbon dioxide evolved passed by convection and diffusion relatively slowly from the surface of the auricle, particularly when this was motionless. Hence it was very important to place the caustic soda receptacle as close as possible to the auricle. When the caustic soda receptacle was within 1 cm. of the auricle the apparatus attained a steady state in 10 minutes, whereas if the caustic soda was 5 cm. above the auricle, the apparatus took more than an hour to attain a steady state.

The cannulæ and manometer bulbs employed are shown in Fig. 1. Type A was used for most experiments on the frog's auricle; type C for experiments on the tortoise's auricle; type B for certain special experiments on the frog's auricle.

The mechanical activity of the auricle was estimated as follows. Cannulæ of known capacity were used. After fluid had been introduced, suction was applied to empty the auricle, and the height reached by the fluid when the auricle was empty was marked. Measurements from this mark gave the diastolic volume and systolic volume of the auricle. The

height of the column of fluid above the auricle gave the diastolic and systolic pressures.

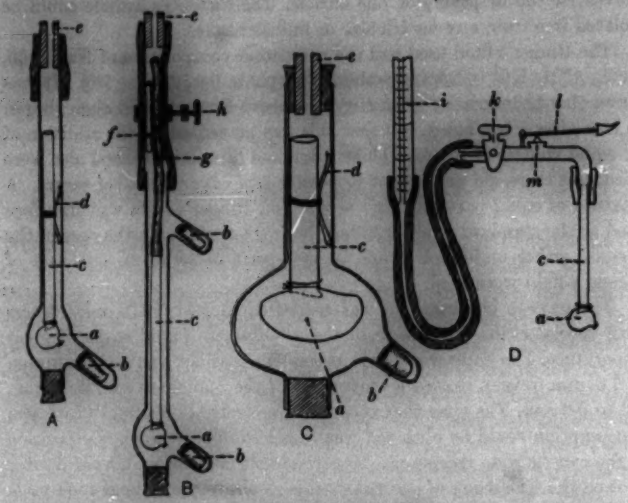

Fig. 1. Apparatus for measuring oxygen consumption and mechanical response of auricle. (Half nat. size.)

A. Bulb for measuring oxygen consumption of frog's auricle.
B. Special bulb for providing auxobaric or isochoric conditions.
C. Bulb for measuring oxygen consumption of tortoise's auricle.
D. Apparatus for measuring isochoric mechanical response at various fillings.

a, auricle; *b*, roll of filter paper soaked in 5 p.c. NaOH; *c*, cannula; *d*, spring to keep cannula in position; *e*, tube leading to Barcroft manometer; *f*, brass tube; *g*, valve tubing; *h*, screw clip; *i*, one c.c. pipette graduated in one-hundredths; *k*, tap; *l*, lever; *m*, membrane manometer.

In the case of the frog's auricle a correction had to be made for the surface tension of the fluid inside the tube. The smallest height of fluid which produced any filling of the auricle was between 1 and 1·5 cm. The height at which filling commenced was measured in each experiment and was subtracted from the pressures observed in order to obtain the true diastolic and systolic pressures. Observation showed that the meniscus of the fluid was unaltered in shape during the excursion, and therefore the action exerted by the surface tension was the same in diastole and systole.

The apparatus permitted the measurement of diastolic and systolic pressures and volumes when the auricle was inside the Barcroft apparatus. The pressures produced during isochoric contraction could not be measured inside the Barcroft apparatus. To obtain these measurements the cannula was taken out and attached to the membrane manometer shown in Fig. 1 D.

The curve of auxobaric activity was determined by introducing varying fillings into the cannula and measuring the diastolic and systolic pressures and fillings.

When making these measurements it was found necessary to keep the auricle exposed to oxygen or to air, and not to immerse it in saline. The auricles of both the tortoise and the frog could maintain undiminished activity for long periods when suspended in oxygen, under both auxobaric and isochoric conditions. The auricles, when suspended in air, also appeared to receive an adequate oxygen supply. For example, no alteration could be detected with certainty in the pressures developed by a frog's auricle, when it was allowed to function under isochoric conditions for an hour suspended in air. When the frog's auricle was immersed in saline and isochoric conditions were maintained there was a rapid decrease in the isochoric response. This was to be expected because the saline oxygenated with air contained only about 7 c.mm. oxygen per c.c. and a frog's auricle used about 0·5 c.mm. oxygen per minute. Hence unless the saline surrounding the auricle was changed continuously the tissue soon suffered from lack of oxygen.

Air was used to fill the Barcroft bulbs in some of our experiments on the frog's auricle, but in most of these experiments and in all the experiments on the tortoise's auricle oxygen was used. The room temperature varied between 12° C. and 15° C. At the end of an experiment the moist weight of the auricle was determined, and, in the case of the frog's auricle, the auricle and the attached bulbar tissue were weighed separately.

The frog's auricle possesses remarkable vitality. A few experiments were performed under aseptic conditions, when the auricle survived and contracted regularly for from 4 to 8 days. Usually readings were confined to the first 12 hours after isolation.

Attempts were made to arrest the auricle by means of a Stannius ligature, and to drive it at a constant rate with an electrical stimulus. We were unable to arrange this in a satisfactory manner. We found, however, that the natural rhythm of the auricle was extremely constant. All experiments in which there was a marked change of frequency were discarded.

Ludwig and Luchsinger(4) stated that the frequency of the frog's heart was increased when the pressure was increased. We found, however, that changes in diastolic pressure and consequently changes in diastolic volume and in oxygen consumption produced no effect on the frequency, and that this remained unchanged both in the frog and the tortoise when the auricle beat for hours at pressures varying from 1 to 10 cm. of water.

The path of the respiratory exchange.

The question as to whether the respiratory exchange of the isolated auricle occurred through its internal or external surface was determined by means of the apparatus shown in Fig. 1 B.

Both the upper and lower receptacles were filled with caustic soda and a short cannula was used, the upper end of which was close to the upper receptacle. The rate of oxygen consumption was measured. Then a piece of valve tubing 15 cm. long was attached to the cannula and passed up to the top of the apparatus, so that the top of this tubing was some 6 cm. above the upper receptacle for caustic. The oxygen consumption remained unaltered. This showed that no measurable amount of CO_2 was liberated from the surface of the fluid in the cannula, for any CO_2 liberated at this surface would have taken at least an hour to diffuse up the tubing and down to the caustic, and during this period the measurements of oxygen consumption would have shown a reduction.

The path of oxygen consumption is proved by the fact that a frog's auricle surrounded by air showed no measurable diminution in force of contraction when made to function under isochoric conditions for an hour, whereas an auricle surrounded by Ringer's fluid showed a 50 p.c. diminution in the pressure it produced after it had functioned thus for 5 minutes. The auricle in air could obtain an adequate supply of oxygen from its surroundings, whereas the auricle immersed in Ringer and contracting isochorically soon exhausted the oxygen dissolved in the fluid in contact with its outside surface.

We concluded therefore that, within the limits of error of our apparatus, the whole of the respiratory exchange of the auricle was independent of the movement of the fluid that it contains.

The auricle is therefore a particularly favourable preparation for the study of the action on the respiratory exchange of cardiac tissue of agencies that interfere with the movement of the tissue, and in particular for the comparison of the respiratory exchange under isochoric conditions with that under auxobaric conditions.

The diffusion of oxygen into the auricle during a steady state.

The frequency of contraction of the auricle is sufficiently great to permit the assumption of a uniform rate of oxygen consumption, and Warburg(5) has provided a formula for calculating the supply of oxygen that can diffuse through a given thickness of tissue. This formula applies to an infinite plane sheet, but the thickness of the frog's auricle (0·1–0·2 mm.) is so small in comparison with its diameter (5–8 mm.) that the curvature may be neglected. In the case of the tortoise the thickness of the auricular wall is an even smaller fraction of the diameter of the auricle. This formula is $d = \sqrt{8c.D/A}$; where d is the thickness in cm. of a section exposed on both sides that will receive an adequate oxygen supply by diffusion: A is the observed oxygen consumption of the tissue in c.c. per g. per minute: c is the concentration of oxygen in atmospheres and D is Krogh's diffusion coefficient for oxygen. This last figure is $1{\cdot}4 \times 10^{-5}$ for muscle at 20° C. and, since it rises 1 p.c. per 1° C. rise of temperature, it is about $1{\cdot}3 \times 10^{-5}$ at 13° C., which was our room temperature.

The volume and weight of the auricle are known, and hence its surface area and the thickness of its wall can be estimated since the auricle is approximately spherical in shape. In the case of the empty auricle the area was measured directly at the end of the experiment. In our experiments the oxygen could only diffuse into the auricle from one side (the

TABLE I. Diffusion of oxygen into tissues.

Weight of organ in g.	Diastolic volume in c.c.	Calculated thickness in cm.	Oxygen consumption in c.c. per g. per minute	Thickness of tissue (d/2) in cm. supplied with adequate oxygen as calculated from Warburg's formula	
				(1) Suspended in air	(2) Suspended in oxygen
(1) Frog's auricle, Exp. 172. Table VIII.					
0·016	0·05	0·025	0·0156	0·018	0·041
—	0·15	0·012	0·0350	0·012	0·028
(2) Frog's auricle, Exp. 234.					
0·024	Empty	0·04	0·0090	0·024	0·053
—	0·3	0·011	0·0300	0·013	0·030
(3) Frog's ventricle. (Data from Fig. 3 in ref. (1).)					
0·130	0·07	0·16	0·0107	0·023	0·052
—	0·2	0·08	0·0180	0·017	0·039
(4) Tortoise's auricle, Exp. C. Table V.					
0·140	Empty	0·04	0·0030	0·040	0·09
—	1·0	0·029	0·0076	0·026	0·058
—	1·9	0·019	0·0133	0·020	0·044

outside), and hence $d/2$ represents the depth that received an adequate oxygen supply.

Table I shows the values obtained with Warburg's formula from typical experiments. These results indicate that the auricles of both the frog and the tortoise, whether full or empty, will receive an adequate oxygen supply when suspended in oxygen, but that when they are suspended in air the oxygen supply will be only just adequate for the filled auricle and will be inadequate for the empty auricle. On the other hand, the figures show that the frog's ventricle, even when suspended in oxygen, will only receive adequate oxygenation through about half the thickness of its wall. These conclusions were tested by direct experiment by measuring the oxygen consumption of the tissues concerned both when suspended in air and when suspended in oxygen.

Oxygen consumption of cardiac tissue in air and in oxygen.

The frog's auricle could not be isolated intact without leaving attached a certain amount of the bulbus arteriosus. A few experiments were made to determine the oxygen consumption of the bulbus arteriosus and of ventricular strips when suspended in air and in oxygen. These results are shown in Table II, together with corresponding figures for the empty but

TABLE II. Observed oxygen consumption of unstretched tissues in air and in oxygen.

	Oxygen consumption in c.c. per g. per hour	
Tissue	(1) Suspended in oxygen	(2) Suspended in air
Frog		
Auricle	0·4–0·8	0·3–0·6
Bulbus arteriosus	0·1–0·2	Nil
Ventricle	0·5–1·0	About 0·1
Tortoise		
Auricle	0·1–0·2	About 0·1
Ventricle	0·3–0·6	About 0·1

contracting auricle. The bulbus arteriosus has a very low oxygen consumption even when suspended in oxygen, and when suspended in air the consumption is too small to measure. An allowance of 0·2 c.c. per g. per hour was made for the oxygen consumption of the bulbar tissue, and the observed figures were corrected in order to obtain the oxygen consumption per unit weight of the auricle.

When unstretched strips of ventricle were suspended in oxygen their oxygen consumption per unit weight was found to be higher than that of the empty auricle. This result is surprising, because the ventricular

strips were quiescent whereas the auricle was contracting. The ventricular strips, when suspended in air, consumed however only about one-fifth as much oxygen as they did when suspended in oxygen. On the other hand, the oxygen consumption of the empty auricle was only reduced about 30 p.c. by the change from oxygen to air, a result in accordance with the calculations from Warburg's formula shown in Table I. The great reduction produced in the oxygen consumption of ventricular strips, when changed from oxygen to air, cannot be accounted for directly by a difference in the rate of diffusion of oxygen, for according to Warburg's formula this change cannot reduce the depth of tissue supplied with oxygen, and therefore the oxygen consumption, more than $\frac{1}{\sqrt{5}}$, *i.e.* $\frac{1}{2\cdot2}$.

The most probable explanation of the excessive reduction in oxygen consumption is as follows. We have shown (1) that acid inhibits the oxygen consumption of cardiac tissue, and probably the portion of the ventricular tissue that is inadequately oxygenated when suspended in air produces sufficient acid to diffuse into and poison the rest of the tissue. Our observations show that strips of cardiac tissue, more than 0·2 mm. thick, do not receive an adequate oxygen supply when suspended in air, even when they are at rest; moreover, even that portion of these strips, which can obtain an adequate oxygen supply, does not consume the normal amount of oxygen. This suggests that all the functions of such strips must proceed in a very abnormal manner, and that all deductions regarding cardiac function made from the study of strips of the cold-blooded ventricle suspended in air must be received with great caution.

In another series of experiments, shown in Table III, the oxygen consumption of the full and empty auricles was determined both in air and in oxygen. The full auricles were filled with a diastolic pressure of 2–3 cm. of water. In these experiments a few milligrams of bulbus arteriosus were

TABLE III. Oxygen consumption of frog's auricles suspended empty and full in air and in oxygen.

	Weight in mg.		Oxygen use of auricular tissue in c.c. per g. per hour			
			Auricle filled		Auricle empty	
Exp.	Auricle	Bulbar tissue	(*a*) In air	(*b*) In oxygen	(*a*) In air	(*b*) In oxygen
194	13	6	1·70	2·25	0·58	0·83
196	12	6	1·81	1·98	0·60	0·55
231	23	11	0·88	0·91	0·36	0·52
232	18	10	2·39	2·61	0·40	0·58
233	18	12	1·15	1·08	0·35	0·59
234	24	9	1·64	1·82	0·33	0·51
		Average	1·60	1·77	0·44	0·59

left attached to the auricle. The oxygen consumption of this tissue was unaffected by the degree of filling of the auricle; it was estimated as 0·1 c.c. per g. per hour for the tissue suspended in air, and as 0·2 c.c. per g. per hour in oxygen. The figures for the oxygen consumption of the auricle were corrected accordingly. This correction was unimportant in the case of the filled auricle, but made a significant difference in the case of the empty auricle.

The averages in Table III show that in the case of the empty auricle the oxygen consumption in air is 25 p.c. less than the oxygen consumption in oxygen, whereas in the case of the full auricle the difference is only 10 p.c.

According to Warburg's formula a tissue that receives an inadequate supply of oxygen when suspended in air, cannot receive more than about 2½ times as much oxygen when it is suspended in oxygen. Since the oxygen consumption of the filled auricle is nearly equal in air and in oxygen, therefore the tissue must get an almost adequate supply of oxygen from air and a completely adequate supply when surrounded by pure oxygen. In the case of the empty auricle the substitution of air for oxygen only reduces the oxygen consumption 25 p.c., and this indicates that the tissue would get a just adequate oxygen supply with an oxygen pressure of about one-half of an atmosphere.

It may be noted that the evidence available indicates that the oxygen consumption of cold-blooded tissues is not increased by an increase in oxygen pressure above that necessary to provide an adequate oxygen supply. For example, Hill(6) found that the resting metabolism of the intact frog at 15° C. was unaltered when the oxygen pressure was varied from 2·8 to 100 p.c. of an atmosphere.

Our figures indicate that the empty auricle, when suspended in oxygen, receives an oxygen supply adequate for its needs, hence the great reduction in oxygen consumption observed, when the auricle is emptied, cannot be due to inadequate oxygen supply.

The effects produced on the oxygen consumption by the substitution of air for oxygen agree with the conclusions derived from Warburg's formula; moreover, they agree with observations made on the general condition of the auricle. An auricle filled with serum and suspended in air can maintain unimpaired activity for more than 24 hours, a fact that indicates that its oxygen supply is very nearly adequate. Similarly, an empty auricle suspended in oxygen does not show any decrease in frequency, and, as soon as it is filled, contracts with full efficiency. On the other hand, an empty auricle suspended in air shows a decrease in rate

after an hour or two, and when filled with serum contracts feebly at first, and only regains its normal activity after several minutes.

The resting metabolism of the frog's heart.

We found (Table II) that the oxygen consumption of a strip of resting frog's ventricle suspended in oxygen was 0·5–1·0 c.c. per g. per hour. Weizsäcker(7) obtained a figure of about 0·1 per hour for the oxygen use of the resting ventricle and we(1) obtained a similar figure when we used his method. With other methods(1) we obtained a figure of from 0·3 to 0·5 for the ventricle, and for the whole heart respectively. We then concluded that the figure of 0·5 was probably nearest the truth, and our present experiments show that even this figure was too low. The oxygen consumption of the resting tortoise's ventricle is slightly lower than that of the frog's ventricle.

The figures given in Table II show that the oxygen consumption of the empty contracting auricle in both the frog and the tortoise is lower than the oxygen consumption of the unstretched resting ventricle.

We made further experiments to see if the oxygen consumption of the frog's auricle was further reduced by arresting it by means of a Stannius ligature. These figures are shown in Table IV, and the averages show that

TABLE IV. Oxygen use of frog's auricle (suspended in oxygen).

		Filled auricle			Empty auricle: Contracting		Empty auricle: Arrested
Exp.	Weight of auricle in mg.	Filling in c.c.	Contractions per minute	Oxygen use in c.c. per g. per hour	Contractions per minute	Oxygen use in c.c. per g. per hour	Oxygen use in c.c. per g. per hour
196	12	0·08	32	1·98	30	0·55	0·42
210	10	0·045	40	1·16	22	0·77	0·48
211	10	0·06	40	1·11	36	0·63	0·36
212	12	0·05	40	1·87	38	0·40	0·14
213	8	—	—	—	40	0·54	0·40
Average	10·4	0·059	38	1·53	33	0·58	0·36

the difference in the oxygen consumption in the empty auricle, when contracting and when resting, is only 0·22 c.c. per g. per hour. On the other hand, the average difference between the oxygen consumption of the auricle, when contracting and filled and when empty and arrested, is 1·17 c.c. per g. per hour.

The oxygen consumption due to the contraction process in the empty auricle is therefore only 19 p.c. of the oxygen consumption due to the

contraction process in the auricle when this is filled at a moderate pressure.

It was rarely possible to produce permanent arrest of the filled auricle by means of a Stannius ligature. In one experiment in which this effect was produced the following results were obtained.

Exp. 196. Frog's auricle. Suspended in oxygen. Weight, auricle 12 mg.; bulbar tissue 6 mg.

Filling in c.c.	Frequency per minute	Oxygen consumption in c.c. per g. per hour
0·085	33	1·98
0	30	0·55
0·06	0	0·68
0	0	0·32

In a later portion of this paper we have calculated the oxygen consumption associated with the contraction process. To obtain this absolutely accurately it would be necessary to determine for each filling studied the oxygen consumption of the contracting and of the resting auricle. It is not practicable to obtain these figures, but the difference found in Exp. 196 between the arrested-filled auricle and the contracting-empty auricle was only 0·13 c.mm. per mg. per hour, whereas the difference between the arrested-filled auricle and the contracting-filled auricle was 1·3 c.mm. per mg. per hour. The assumption that the oxygen consumption of the contracting-empty auricle indicates the oxygen consumption of the resting-filled auricle involves therefore an error of 10 p.c. or less in the calculation of the oxygen consumption associated with the contraction process.

We obtained similar results with the frog's heart and the frog's ventricle (1), for in these cases we found that within the limits of error the oxygen consumption of the empty contracting organ was the same as that of the filled and arrested organ.

We measured the oxygen consumption of the empty frog's auricle suspended in oxygen in 17 experiments. The average figure was 0·53 c.c. per g. per hour. In the case of the tortoise's auricle eight similar experiments gave an average of 0·14 c.c. per g. per hour. In both cases a remarkable individual variation was observed. In the case of the frog the figures varied from 0·31 to 0·9 c.c. per g. per hour, and in the case of the tortoise from 0·11 to 0·19 c.c. per g. per hour. A considerable variation is to be expected, for Hill (6) found that the basal metabolism of a frog kept in captivity decreased 50 p.c. after 10 days' starvation, and Bohr (8) found that after 12 days' captivity the respiratory exchange of

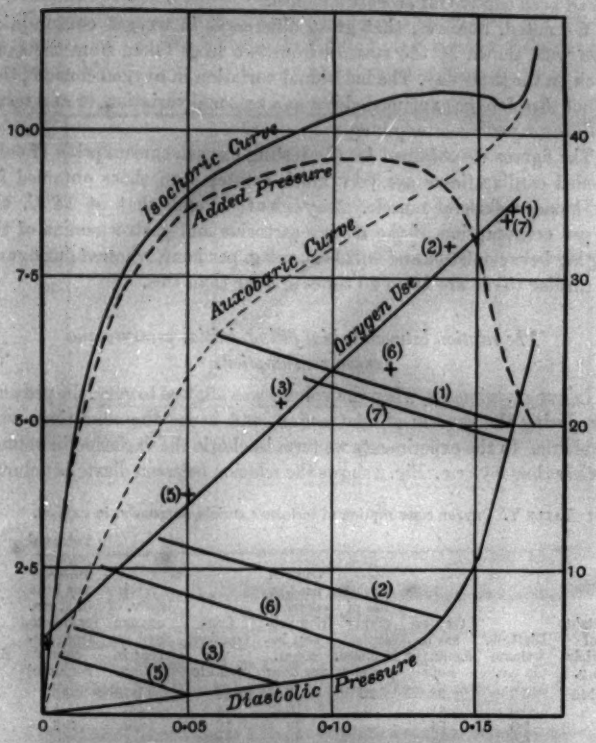

Fig. 2. Influence of diastolic filling on mechanical response and oxygen consumption of the frog's auricle.

Exp. 172. Auricular weight 16 mg. Auricle filled with frog's serum and Ringer's fluid equal parts. Frequency 38 per min.

Abscissa. Diastolic filling in c.c.

Ordinates. Left, pressure in cm. water; right, oxygen use in c.mm. per hour.

The numbers indicate the order in which the measurements were taken. The straight lines indicate the auxobaric response of the auricle at various fillings.

a frog decreased to one-quarter of its original value. The frogs used by us had been captive for at least a month.

We noted, however, that great differences in oxygen consumption often were shown by the auricles from two frogs taken from the same batch on the same day. The individual variation in oxygen consumption cannot therefore be explained either as a seasonal variation, or as a result of different periods of captivity.

The figures we obtained for the resting oxygen consumption of cold-blooded cardiac tissue are very much greater than those obtained for cold-blooded skeletal muscle. Meyerhof(9) found that at 14° C. the oxygen consumption of the resting sartorius and gastrocnemius of the frog lay between 0·014 and 0·028 c.c. per g. per hour, whereas our figures for cardiac tissue are 10 to 20 times greater than this.

The relation between initial filling, initial pressure and oxygen consumption.

In our experiments, when the volume was allowed to vary, the pressure rose during the process of contraction, and hence the conditions were auxobaric. In the experiments we term isochoric the variation in volume was less than 0·01 c.c. Fig. 2 shows the relation between diastolic volume,

TABLE V. Oxygen consumption of tortoise's auricle, suspended in oxygen.

Exp.	Weight of auricle in g.	Diastolic volume in c.c.	Oxygen use in c.mm. per hour	Oxygen use of empty auricle in c.mm. per hour	Oxygen used in contraction process in c.mm. per hour	Contractions per minute	Work equiv. of oxygen used per beat in g. cm.	Potential energy released per beat in g. cm. (calculated from isochoric response)	Efficiency p.c.
C	0·140	0·3	32	25	7	14	1·78	0·5	28
		0·6	41	—	16	14	4·0	1·5	37·5
		1·0	64	—	39	16	8·6	3·3	39
		1·6	95	—	70	16	15·5	6·2	38
		1·9	111	—	86	16	19·2	7·5	39
D	0·158	0·48	45	30	15	17	3·06	0·11	3
		0·96	56	—	26	—	5·4	0·50	9
		1·4	66	—	36	—	7·5	1·2	16
		1·75	93	—	63	—	13·1	1·5	12
		2·6	125	—	95	—	19·8	3·8	19
		3·2	135	—	105	—	21·7	6·0	28
		3·4	142	—	112	—	22·3	6·8	31
F	0·173	2·64	232	24	208	20	36·8	20·2	55
H	0·099	1·65	60	13	47	12·5	12·9	4·9	38
I	—	0·6	34·4	18	16·6	20	2·4	0·7	29
J	0·114	1·2	78	18	60	17·5	12·1	2·4	20
K	0·090	1·7	80	10	70	16	15·5	7·8	50

diastolic pressure, systolic pressure under auxobaric and under isochoric conditions, and oxygen consumption of the frog's auricle.

The relation between the diastolic filling and the oxygen consumption of the tortoise's auricle is shown in Table V, and some of these data are plotted in Fig. 3.

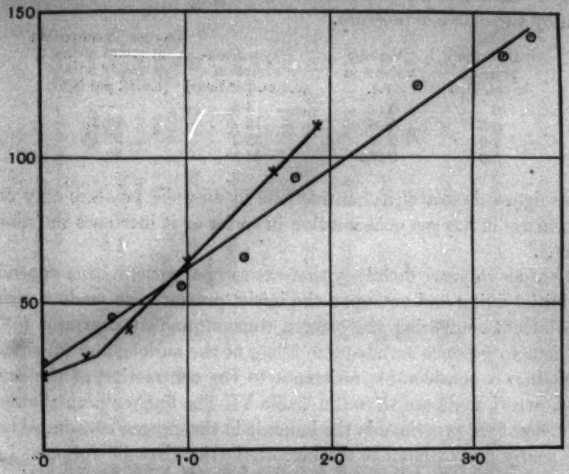

Fig. 3. The relation between diastolic volume and oxygen consumption of the tortoise's auricle.

Ordinate. Oxygen consumption in c.mm. per hour.
Abscissa. Diastolic volume in c.c.
The figures are taken from Table V; crosses = Exp. C; circles = Exp. D.

Figs. 2 and 3 both show a linear relation between diastolic filling and oxygen consumption. This result agrees with our previous finding with the frog's ventricle. Our results with the frog's ventricle might, however, have been due in part or wholly to a deficiency in oxygen supply, for if the oxygen supply of a tissue is deficient, then any increase in surface will increase the oxygen consumption by increasing the supply of oxygen diffusing into the tissue. This objection does not apply in the case of the experiments described in this paper, for we have shown that in the case of the frog's auricle the oxygen supply of the tissue, when it is suspended in oxygen, is more than adequate both when the auricle is filled and when

it is empty. We did not repeat these tests on the tortoise's auricle because the calculations shown in Table I indicate that the tortois auricle is less likely to suffer from oxygen deficiency when suspended oxygen than is the frog's auricle.

The following results, taken from Fig. 2, show clearly that the rate of oxygen consumption is determined by the volume of the auricle rather than by the diastolic pressure.

Diastolic pressure in cm. H_2O	Diastolic volume in c.c.	Oxygen consumption (c.mm. per hour)	Oxygen consumption in excess of that of empty auricle (c.mm. per hour)
0	0	4·8	—
0·3	0·05	15	10·2
1·6	0·14	32·2	27·4
5·0	0·163	34·8	30

These figures indicate that an increase in diastolic pressure only causes an increase in oxygen consumption in so far as it increases the diastolic volume.

In order to prove definitely that the oxygen consumption depends on the initial filling and not upon the initial pressure, we made a series of experiments comparing the oxygen consumption at pressures (*a*) just sufficient to produce an adequate filling of the auricle, and (*b*) sufficient to produce a considerable resistance to the contraction of the auricle. These experiments are shown in Table VI. The final column shows that in the first four experiments the increase in the oxygen consumption was very nearly proportional to the increase in the diastolic volume. In the last four experiments the percentage increase in oxygen consumption was distinctly less than the increase in diastolic volume.

Fig. 2 shows that increase of diastolic pressure at first causes an increase in initial volume and an increase in the added pressure, but that when the diastolic pressure rises above 1 cm. water the rate of increase of volume diminishes and the added pressure falls. A diastolic pressure of 1 cm. of water may therefore be termed the optimum pressure for this auricle. This optimum pressure naturally varies in different preparations, but is usually between 1 and 3 cm. of water. Fig. 2 shows that a rise in diastolic pressure up to a point slightly above this optimum pressure causes an increase in oxygen consumption proportional to the increase in diastolic volume, although not proportional to the rise in pressure. The last four experiments in Table VI, however, show that when still higher diastolic pressures are applied the oxygen consumption does not even increase in proportion to the increase in diastolic volume. This suggests that auricular muscle resembles skeletal muscle in that overstretch-

TABLE VI.

	Low pressures						High pressures						Ratios		
No. of exp.	Diastolic pressure in cm. H_2O	Added pressure in cm. H_2O	Diastolic volume in c.c. (D.V. 1)	Excursion in c.c.	Oxygen use in c.mm. per hour	Oxygen use less O_2 use of empty heart (Ox. 1)	Diastolic pressure in cm. H_2O	Added pressure in cm. H_2O	Diastolic volume in c.c. (D.V. 2)	Excursion in c.c.	Oxygen use in c.mm. per hour	Oxygen use less O_2 use of empty heart (Ox. 2)	A D.V. 2 / D.V. 1	B Ox. 2 / Ox. 1	B/A
172	1·7	8	0·14	0·10	32·2	27·4	5	6	0·165	0·105	34·8	30	1·17	1·10	0·94
	0	—	—	—	4·8	—	—	—	—	—	—	—	—	—	—
171	2·6	7	0·200	0·07	19·4	15·2	5·5	7	0·200	0·044	19·3	15·1	1·00	1·01	1·01
	0·5	6·5	0·130	0·07	13·4	9·2	—	—	—	—	—	—	1·54	1·64	1·06
	0	—	—	—	4·2	—	—	—	—	—	—	—	—	—	—
125*	2·5	7	0·08	0·06	9·2	(6·2)	6·2	4	0·110	0·06	11·8	(8·8)	1·38	(1·41)	(1·02)
153*	3·1	8	0·14	0·087	10·9	(7·9)	7·5	3	0·180	0·039	12·4	(9·4)	1·28	(1·19)	(0·94)
	1·0	8	0·11	0·072	9·3	(6·3)	—	—	—	—	—	—	1·63	(1·49)	(0·91)
114	2·0	7·5	0·180	0·09	16·6	12·6	6·0	4	0·180	0·06	15·2	11·2	1·00	0·88	0·88
	0	—	—	—	4·0	—	—	—	—	—	—	—	—	—	—
129*	2·0	6·3	0·073	0·04	11·4	(8·4)	6·2	3	0·080	0·025	10·4	(7·4)	1·11	(0·88)	(0·88)
167	2·5	7	0·056	0·032	9·7	6·7	10·0	2·5	0·09	0·012	10·3	7·3	1·60	1·09	0·68
	0	—	—	—	3·0	—	—	—	—	—	—	—	—	—	—
189	2·6	4·4	0·175	0·12	33·4	23·6	8·5	4·0	0·18	0·02	27	17·2	1·03	0·73	0·71
	0	—	—	—	9·8	—	—	—	—	—	—	—	—	—	—

* In Exps. 125, 153 and 129 the oxygen use of the empty auricle was not measured; it was estimated at 3 c.mm. per hour and the figures derived from this estimate are put in brackets.

ing reduces its oxygen consumption. Unfortunately, it was impossible to produce gross overstretching of the auricle by employing pressures greater than 10 cm. of water because such pressures caused leakage of the auricular contents.

The oxygen consumption under auxobaric and isochoric conditions.

Our experiments on the frog's ventricle showed that increased resistance to output without change of diastolic volume did not cause any increase in oxygen consumption, but that when such resistance interfered with the heart's output it caused a decrease in the oxygen consumption. This effect might, however, have been due to interference with the oxygen supply of the ventricle. The measurements of the oxygen consumption of the frog's auricle, under isochoric conditions, are free from this objection because this tissue is not dependent for its respiratory exchange on the movements of the fluid which it contains.

The chief experimental difficulty was the fact that isochoric conditions often caused the frog's auricle to leak. This reduced the diastolic pressure and the diastolic filling and therefore caused a reduction in the oxygen consumption. The auxobaric and isochoric activities of the frog's heart were studied in the apparatus shown in Fig. 1 B. In these experiments the apparatus was filled with oxygen. Eleven experiments were performed, and the details of five experiments are shown in Fig. 4 and the average results in Table VII. In every case the introduction of isochoric

TABLE VII.

		Average oxygen use in c.mm. per hour	
		5 exps. shown in Fig. 4	7 other exps.
Period 1.	Auxobaric	23·6	20
„ 2.	Isochoric	21·2	17
„ 3.	Auxobaric	20·8	17

conditions caused a diminution in the oxygen consumption. Great care was of course taken to see that the heart volume under isochoric conditions was the same as the diastolic volume under auxobaric conditions.

We have previously shown that, within the limits of our experimental error, the whole of the oxygen consumption occurs through the wall of the auricle and therefore the reduction in oxygen consumption is not due to any interference with the respiratory exchange. Moreover, it is not due to injury of the auricle by accumulation of metabolic products, because control experiments showed that the systolic pressures produced by the auricle did not alter when it was made to function for an hour

under isochoric conditions. Fenn(10) showed that when a muscle did work the amount of energy liberated was greater than that liberated

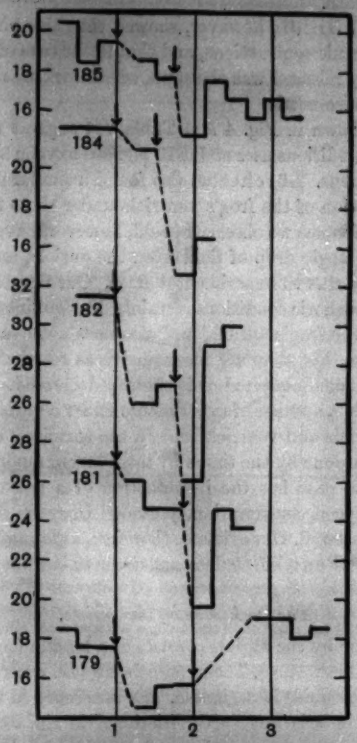

Fig. 4. Oxygen consumption of frog's auricle under auxobaric and isochoric conditions.

Abscissa. Time in hours.

Ordinate. Oxygen consumption in c.mm. per hour.

Isochoric conditions were introduced between the times marked by the arrows. Five experiments are shown (179–185).

under isometric conditions. The excess of energy was approximately equal to the work done, and approached zero both when the muscle contracted against a very low resistance and when the resistance became

too great for the muscle to overcome. When maximum work was done the energy released was about 20 p.c. greater than that released under isometric conditions.

Hartree and Hill(11), however, showed that this phenomenon only occurred with tetanic contractions, and that in the case of a single muscle twitch the energy released was the same when work was done and when the response was isometric.

The results shown in Fig. 4 and Table VII suggest that the auricle under isochoric conditions uses about 10 p.c. less oxygen than when under auxobaric conditions. Lüscher(12) also found a similar difference in the oxygen consumption of the frog's ventricle under these two conditions.

The small difference we observed could, however, have been produced by the leak of a single drop of fluid from the auricle, and therefore the difference may be due to experimental error. Our figures show that the introduction of isochoric conditions certainly does not increase the oxygen consumption of the frog's auricle, and also does not cause a decrease of more than 10 p.c., but they are inconclusive as to whether the oxygen consumption remains unaltered or is slightly decreased.

We have made a number of experiments under a variety of conditions on the frog's auricle and ventricle and on the tortoise's auricle to try to increase the work done by the tissue by introducing moderate resistances to output. In no case has the introduction of a resistance to output increased the oxygen consumption, provided that the diastolic volume has remained unaltered. Our evidence therefore, as far as it goes, supports the view that the Fenn effect does not occur in cardiac tissue.

The work done by the auricle.

The work done by the auricle consists of a kinetic factor and a static factor (output × resistance). The kinetic factor (*i.e.* work done in imparting velocity to the fluid) is negligible. The resistance in the experiments was chiefly gravimetric but partly frictional in the case of the frog's auricle. We measured the duration and volume of output of the frog's auricle and calculated the velocity of output, and it was found that pressures between 0·5 and 1·0 cm. of water produced velocities of outflow comparable to those produced by the auricle. The frictional resistance therefore was not great, but it was sufficient to account for about one-third of the differences in pressure observed between the auxobaric and isochoric readings of the frog's auricle. In the case of the tortoise's auricle the frictional resistance was negligible.

Our method of calculating work done is shown in Fig. 5. The auricle

under a diastolic pressure of 2·6 cm. water has a diastolic volume of 0·083 c.c.; and on contraction it expels 0·06 c.c. and the systolic pressure

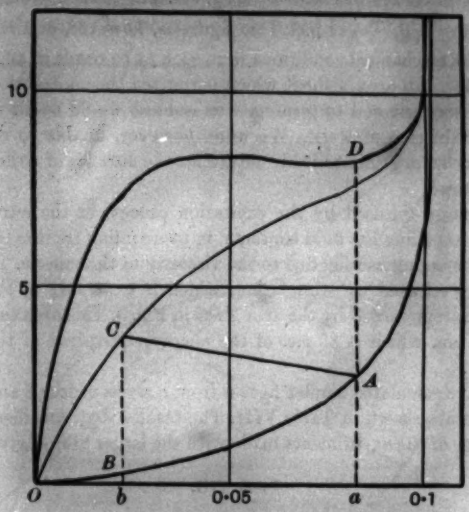

Fig. 5. The mechanical response of the frog's auricle.

Abscissa. Diastolic filling in c.c.
Ordinate. Pressure in cm. H_2O.

The curves from below upwards show (1) diastolic pressure, (2) systolic pressures produced by auxobaric contraction, (3) systolic pressures produced by isochoric contraction. The line *AC* shows the auxobaric contraction when the diastolic filling is 0·085 c.c.

rises to 3·7 cm. The work done is therefore represented by the area *aACb*, but of this amount the area *aABb* is due to the elasticity of the auricle wall and therefore the work performed by the contraction is represented by the area *ACB*. The output is measured in c.c. and the resistance in g. pressure per sq. cm., and the area *ACB* corresponds to 0·12 g. cm. work. If the frictional resistance is equivalent to 1 g. per sq. cm. pressure, then the work done becomes 0·18 g. cm. The auricle with a volume of 0·083 c.c. and a frequency of 35 per minute used 19 c.mm. oxygen per hour, but of this 3 c.mm. must be subtracted as representing resting metabolism. The oxygen consumption per beat due to the contraction process was $\frac{19-3}{35\times 60} = 0{\cdot}0076$ c.mm.

If the consumption of 1 c.c. oxygen corresponds to 5 calories and 1 calorie = 42,400 g. cm., then 1 c.mm. oxygen gives 212 g. cm. Hence the release of energy per beat = 1·61 g. cm. The efficiency of the auricle is therefore $\frac{0{\cdot}18 \times 100}{1{\cdot}61} = 11$ p.c. This figure is, however, of little interest because the mechanical conditions were such as to result in an inefficient performance. An arrangement which permitted the contraction to commence as isochoric and to terminate as isobaric would result in a much greater production of work. We were, however, unable to devise any satisfactory arrangement of this nature for the auricles of either the frog or the tortoise.

The energy released by the excitation process in the auricle is expended in expelling the fluid contents, in overcoming friction in the fluid and in overcoming friction due to the viscosity of the muscle. The potential energy released by stimulation, which is expended in these three manners, is represented by the area *ADO* in Fig. 5. This area corresponds to 0·53 g. cm. which is 33 p.c. of the energy equivalent of the oxygen consumed.

We have calculated similar figures from a series of frog's auricles and the results are shown in Table VIII. The results vary considerably, but the average of the experiments made with the larger fillings gives a value

TABLE VIII.

	1	2	3	4	5	6	7	
Exp.	Diastolic volume in c.c.	O_2 use in c.mm. per hour	Basal oxygen use in c.mm. per hour	Oxygen used in contraction (1–2)	Contractions per min.	O_2 use per beat in c.mm.	Work equiv. of O_2 used in g. cm.	Potential energy released per beat in g. cm. calculated from isochoric response	Theoretical efficiency in p.c.
189	0·28	55	—	45·2	38	0·0198	4·22	1·38	33
	0·15	26·6	—	16·8	36	0·0078	1·66	0·50	30
	0·10	25·5	9·8	15·6	40	0·0065	1·73	0·32	20
188	0·12	23·5	7·7	15·8	36	0·0073	1·55	0·85	55
	0·032	12·2	—	4·5	30	0·0025	0·53	0·14	27
185	0·085	19	3	16	35	0·0076	1·63	0·53	33
184	0·12	22	5	17	32	0·0088	1·88	0·56	30
181	0·112	27	5	22	32	0·0114	2·45	0·92	38
180	0·10	28	5	23	37	0·0104	2·21	0·85	38
179	0·125	17·6	3	14·6	40	0·0061	1·30	0·63	48
177	0·144	30·2	6	24·2	43	0·0094	2·02	0·91	45
176	0·15	19·6	7·2	12·4	23	0·009	1·92	1·29	67
175	0·05	16	6	10	32	0·0054	1·12	0·32	29
172	0·154	34·4	4·8	29·6	38	0·013	2·78	1·31	47
	0·14	32	—	27·2	—	0·0124	2·56	1·16	44
	0·08	21	—	16·2	—	0·0071	1·52	0·56	36
	0·05	15	—	10·2	—	0·00445	0·955	0·32	33

of the potential energy released as 42 per cent. of the energy equivalent of the oxygen consumed.

The relation between the oxygen consumption and the work done in the tortoise's auricle is shown in Table V and Fig. 6. In the experiment

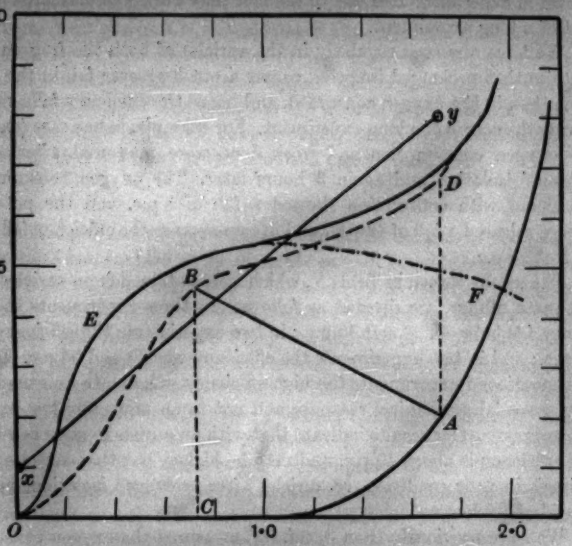

Fig. 6. The mechanical response and oxygen consumption of the tortoise's auricle.

Exp. K (Table V). Auricular weight 0·090 g. Frequency 16 per min.

Abscissa. Diastolic filling in c.c.

Ordinate. Pressure in cm. water, or oxygen consumption in 0·01 c.c. per hour.

The following measurements are shown; *OCA*, diastolic pressure; *OBD*, systolic auxobaric pressure; *OED*, systolic isochoric pressure; *OEF*, added systolic isochoric pressure; *x–y*, oxygen consumption; *A–B*, course of auxobaric contraction at 1·7 c.c. diastolic filling; *ABC*, work done; *ADEO*, potential energy released.

shown in Fig. 6, when the diastolic filling was 1·7 c.c., the oxygen consumption due to contraction was 80 − 10 = 70 c.mm. oxygen per hour. The frequency was 16 per minute and the work equivalent of the oxygen used per beat was 15·5 g. cm. The work done per beat (Fig. 6, area *ABC*) was 3·1 g. cm. which gives an efficiency of 20 p.c., and the potential energy

released per beat (Fig. 6, area *ADEO*) was 7·8 g. cm. which corresponds to an efficiency of 50 p.c. Table V shows seven experiments on the tortoise's auricle in which the work equivalent of the oxygen consumed and the potential energy released per beat were calculated. The low efficiency shown in experiment D is due to the fact that work was measured at the end of a long experiment.

We have observed regularly in the auricles of both the frog and the tortoise that prolonged isolation causes a much greater fall in the work done than in the oxygen consumed, and hence the efficiency falls greatly during the course of a long experiment. For example, in one case (exp. K) the oxygen consumption and work done were measured after about 2 hours' isolation and again 3 hours later. The oxygen consumption associated with contraction showed a fall of 8 p.c. and the potential energy released a fall of 42 p.c., and in consequence the efficiency fell from 50 to 32·5 p.c.

The results shown in Table V, which were obtained from auricles with adequate fillings, are divided as follows. In three experiments the efficiency fell between 20 and 30 p.c.; in two experiments the efficiency was 38 p.c.; and in two experiments the efficiency was 50 and 55 p.c. In the last-mentioned experiments the high efficiency was due to an exceptionally powerful mechanical response and not to an unusually low oxygen consumption. Our results indicate that with an auricle in good condition the efficiency is about 40 p.c. and may be higher, but that auricles from animals in poor condition, or auricles after prolonged isolation, have a considerably lower efficiency.

We have previously (Fig. 3, ref. (1)) measured the oxygen consumed and the work performed by the frog's ventricle, and in this case the potential energy released per beat is 56 p.c. of the work equivalent of the oxygen consumption associated with contraction.

The theoretical efficiency in skeletal muscle is 40 per cent., and our results suggest that this value for cardiac muscle is similar but possibly somewhat higher. The results obtained with the frog's auricle (Table VIII) show that the theoretical efficiency is considerably lower with small than with large fillings, and the results obtained with the tortoise's auricle (Table V) also show a rise of efficiency with increased filling.

The simplest method of measuring the theoretical maximum work of the auricle is to plot the added pressure against the diastolic volume. Such curves are shown in Figs. 2 and 6. Inspection of these curves shows that, when the diastolic pressure is fairly near to that which produces the optimum diastolic filling, the multiplication of the diastolic volume (in

c.c.) and the added pressure (in g. per sq. cm.) give a figure which is within about 10 p.c. of the theoretical maximum work. Hence if the diastolic volume is not changed, but the auricular response is changed by drugs, etc., then the theoretical maximum work varies very nearly as the added pressure.

In previous papers(1,2) we calculated the influence of ionic changes on work and oxygen consumption by plotting the added pressure produced at a fixed diastolic volume against the oxygen consumption produced at this volume. The results in the present paper indicate that this method, although imperfect, gave a roughly quantitative estimate of the variations that occurred in the potential energy released.

Discussion.

The study of the oxygen consumption of the auricles of the frog and the tortoise has enabled us to eliminate certain possible sources of error which rendered uncertain some of the conclusions based on our work on the frog's ventricle. In our work we have continually had reason to be impressed with the ease with which the activity of cardiac tissue is impaired by any process that interferes with its respiratory exchange. This source of error can be easily avoided with the auricle, owing to the fact that it can maintain an adequate respiratory exchange through its external wall. The work on the auricle has, however, confirmed all our previous conclusions regarding the oxygen consumption of the ventricle. Our results show that the resting metabolism of cardiac tissue is from ten to twenty times as great as that of skeletal tissue. This difference is in accordance with the difference in function of the two tissues. The skeletal muscle has prolonged periods of inactivity whereas the cardiac muscle never rests. A low resting oxygen consumption is an important economy in the former case, but is of no importance in the latter case. In the auricle, as in the ventricle, the degree of diastolic filling is by far the most obvious factor that influences the oxygen consumption. This fact is particularly obvious in the case of the tortoise's auricle, and Fig. 3 shows examples in which the oxygen consumption of an auricle was varied five- and even seven-fold by variations in the filling.

Weizsäcker(13), Bodenheimer(14), Lüscher(12), working with the frog's heart, and Starling and Visscher(15), using the dog's heart, all found a great variation in oxygen consumption when the filling was varied. Riesser(16) has recently reported that the lactic acid production of frog's hearts poisoned by cyanide varies as the diastolic filling of the heart, but is not influenced by the resistance against which the heart con-

tracts. It is true that some of the former authors thought that the oxygen consumption was influenced more by variations in diastolic pressure than by variations in diastolic filling, but these two factors tend to vary together, and hence it is not easy to distinguish their relative importance.

As a general rule our experimental data agree with the data obtained by these workers, although our interpretation differs in some cases. On the other hand, Bohnenkamp and his co-workers (17), in a series of papers, have advanced the view that the energy liberated per beat by the frog's ventricle is constant and is not influenced by variations in pressure or in filling. For example, Bohnenkamp, Eismayer and Ernst (18) measured the oxygen consumption of the frog's ventricle and concluded that this was the same with zero diastolic pressure and with high diastolic pressures. This conclusion is directly opposed to the results of all the experiments we have made on a considerable variety of preparations of cold-blooded cardiac tissue. Eismayer and Quincke (19) have however recently published results which are in general agreement with our findings, and in particular confirm the linear relation between the diastolic volume and the oxygen consumption of the frog's ventricle.

Our results show that over a wide range of fillings there is a linear relation between the oxygen consumption due to the process of contraction and the initial volume of the auricle. There cannot, however, be any direct relation between these two sets of figures, and therefore we have examined our results to see if any relation is apparent between the surface area of the auricle and its oxygen consumption.

We measured the surface area of empty auricles by cutting them open and laying them out flat. This method showed that the surface area of the unstretched frog's auricle varied from 0·4 to 0·8 sq. cm. and that of the unstretched tortoise's auricle varied from 3·5 to 4·5 sq. cm. We calculated the surface area of auricles filled with known volumes by assuming that they were spherical and using the formula $\div$ surface $= 4{\cdot}83\ (\text{vol.})^{\frac{2}{3}}$. The results thus obtained were inconclusive, as there was an approximately linear relation between surface area and oxygen consumption with both moderate and large fillings, but the oxygen consumption of the empty auricle was considerably lower than would accord with a linear relationship. We have previously shown, in Table VI, that over-stretching of the heart with an excessive filling reduces its oxygen consumption, and hence the relation between surface area and oxygen consumption cannot be linear above a certain optimum filling.

The oxygen consumption of the auricles of the frog and of the tortoise per unit weight and unit surface is shown below.

	Frog's auricle	Tortoise's auricle
Oxygen use of empty auricle in c.c. per g. per sq. cm. surface	0·75–1·1	0·04–0·05
Increment in oxygen use (c.c. per g.) per sq. cm. increase of surface	0·8 –1·9	0·09–0·15

These figures show that the oxygen consumption per unit of weight and surface is more than ten times greater in the frog than in the tortoise. This difference cannot be accounted for by differences in frequency because the frequency of the frog's auricle is less than three times that of the tortoise's auricle.

The figures also show that the oxygen use per unit of surface in the empty auricle is considerably less than the increment in oxygen consumption associated with unit increase of surface as caused by increased filling. This fact supports the view, which we have previously put forward, that there is a fundamental difference between the resting metabolism and the metabolism associated with the contraction process.

Conclusions.

1. Methods are described for measuring the oxygen consumption of the isolated auricles of the frog and of the tortoise.

2. The auricles of the frog and tortoise are sufficiently thin for full respiratory exchange to occur by diffusion through their external surface. Hence the respiratory exchange is not affected by interference with the movement of the fluid inside the auricle.

3. The resting metabolism of cardiac tissue is much higher than that of skeletal muscle. The following are the approximate figures in c.c. oxygen per g. per hour. Frog: auricle 0·4–0·8, ventricle 0·5–1·0. Tortoise: auricle 0·1–0·2, ventricle 0·3–0·6.

4. The oxygen consumption of the auricle increases with increase of diastolic volume. The maximum oxygen consumption of the extended auricle may be five times that of the empty auricle. The oxygen consumption is only affected by initial pressure in so far as this affects the initial volume. Increase of initial pressure above a certain figure reduces the oxygen consumption.

5. The oxygen consumption under isochoric conditions is either equal to, or possibly slightly less than, the oxygen consumption under auxobaric conditions.

6. The potential energy released on contraction can be calculated from the pressure-volume curves. This gives a value of about 40 p.c. of the work equivalent of the oxygen consumed. This indicates that the

relation between energy released in the initial contraction process and in the recovery process is similar in the frog's heart to that found in skeletal muscle.

7. The actual work performed by the heart in our experiments was small owing to the mechanical inefficiency of the conditions obtaining in our apparatus. The highest value obtained was 20 p.c. of the work equivalent of the oxygen consumed.

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ACTION OF IONIC CHANGES ON THE OXYGEN CONSUMPTION OF THE FROG'S AURICLE.

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We have previously (1) measured the action of various ionic changes and of ethyl urethane on the oxygen consumption and on the mechanical response of the frog's ventricle. Various writers had brought forward evidence that such influences could abolish the mechanical response without greatly reducing the oxygen consumption. This evidence is discussed in our previous paper (1). We found that in the frog's ventricle these two factors were always reduced in equal proportion.

Our results with the frog's ventricle are subject to certain errors. The chief of these is that the ventricle is too thick to permit of an adequate oxygen supply being provided by diffusion through its outside wall, hence the ventricle when arrested or when beating very feebly must suffer a partial oxygen want. We have shown (2) that the auricle is free from this objection because when filled with fluid the oxygen supply it receives by diffusion through its outside wall is nearly adequate for its maximum activity even when it is suspended in air, and is fully adequate when it is suspended in oxygen. Hence the auricle is a much better preparation than is the ventricle on which to study the oxygen consumption when the tissue is paralysed.

Our previous experiments have shown that both the ventricle and auricle have an oxygen consumption when resting that is far higher than that of resting skeletal muscle. We have shown that this resting metabolism is but little affected by depressant drugs, and hence such drugs when they arrest the mechanical response do not abolish the oxygen consumption but reduce it to the level of the resting unpoisoned tissue. The change in the mechanical response produced by drugs must therefore be compared, not with the change in the total oxygen consumption, but with the change in the oxygen consumption associated with the contraction process.

Methods.

We have repeated upon the frog's auricle the experiments previously made on the frog's ventricle. Our chief purpose has been to determine with certainty the oxygen consumption of the auricle when completely or nearly completely paralysed, so as to see whether the oxygen consumption under such conditions is similar to the oxygen consumption of the resting tissue, or whether it is possible to paralyse the auricle without reducing the oxygen consumption to this level.

Our estimations of the work performed by the frog's ventricle were unsatisfactory, for we measured the pressure added during the isochoric response. In this paper we have estimated the potential energy released by the contraction process, according to the method previously described (2).

Unfortunately, the normal auricle is a fragile tissue and we found that, when the auricle was partially paralysed, its response was so feeble that it could not be measured accurately. Our estimations of the potential energy released were therefore only approximately accurate, but they indicated roughly the degree in which the mechanical response of the auricle was depressed.

The methods used for measuring the oxygen consumption and mechanical response of the auricle have been described in a previous paper (2).

The fluid used to fill the auricle was a mixture of equal portions of frog's serum and Ringer's fluid, except when otherwise stated. The work equivalent of the oxygen consumed was calculated from the figure 1 c.mm. O_2 = 212 g. cm. work. The potential energy released per beat by the auricle was calculated from the curves showing the diastolic and systolic pressures produced by the isochoric response at varying fillings. The oxygen consumption observed required correction for the presence of a few mg. of bulbus arteriosus, left attached to the auricle. This extra tissue was not affected by the depressants introduced into the auricle, and hence it was important to subtract its oxygen consumption before estimating the effect on oxygen consumption produced by the drug.

We have shown (2) that the oxygen consumption of the bulbus arteriosus, suspended in oxygen is about 0·2 c.c. per g. per hour. At the end of each experiment the auricle and extra tissue were weighed separately (moist weight), and the oxygen consumption of the bulbus was subtracted from the observed oxygen consumption. We have also shown previously (2) that the average oxygen consumption of the empty frog's auricle

suspended in oxygen is 0·53 c.c. per g. per hour, but that wide individual variations may occur in this figure.

Action of ethyl urethane.

Concentrations of urethane sufficient to reduce the mechanical response of the auricle always reduced its frequency, and hence no exact comparison could be made between the work done and oxygen used. The following are the figures obtained in an experiment in which the auricle was suspended in oxygen.

Exp. 202. Weight of auricle 11 mg.

Fluid	Diastolic filling in c.c.	Frequency per min.	Oxygen used in c.mm. per hour (*a*) Total	(*b*) Minus basal metabolism	Work equiv. of oxygen used (*b*) in g. cm. per beat	Potential energy released in g. cm. per beat
Serum + Ringer	0·10	50	29·4	20·4	1·4	0·65
Empty	0	30	9	—	—	—
0·22 molar ethyl urethane added	0·10	24	11	2·0	0·3	0·1–0·2
0·35 molar ethyl urethane added	0·09	0	6·4	—	—	—

This experiment shows that the oxygen consumption after the auricle was arrested with urethane was 0·58 c.c. per g. per hour, whereas the oxygen consumption of the empty contracting auricle was 0·82 c.c. per g. per hour. We have shown (2) that the average oxygen consumption of the empty auricle arrested by a Stannius ligature is 0·22 c.c. per g. per hour less than that of the empty contracting auricle. Hence the difference in oxygen used by the empty unpoisoned auricle and by the urethanized auricle can be accounted for by the arrest of the auricle in the latter case. The experiment also shows that a concentration of urethane sufficient to reduce the mechanical response of the heart to $\frac{30 \times 0{\cdot}15}{50 \times 0{\cdot}65} = \frac{1}{7}$ also reduces the oxygen consumption due to contraction to $\frac{2}{20} = \frac{1}{10}$. Other experiments gave similar results and we concluded that the drug depressed the mechanical response and the oxygen consumption associated with contraction equally, but that concentrations of urethane sufficient to arrest the auricle did not depress the basal metabolism of the tissue.

A few experiments were made with ethyl urethane in concentrations between 0·1 and 0·2 molar. These produced no certain reduction in either the oxygen consumption or in the potential energy released. Hence urethane in concentrations too low to reduce the mechanical response does not reduce the oxygen consumption.

Action of excess of potassium.

The concentration of potassium chloride in the normal serum-Ringer mixture was about 0·016 p.c. Experiments made in air showed that a concentration of 0·06 p.c. was sufficient to reduce the frequency to one-quarter of the normal, and that there was a corresponding fall in the oxygen consumption. Other experiments made in air, which are summarized in Table I, show that concentrations of 0·01 p.c. and more of KCl arrested the auricle but did not reduce the oxygen consumption below that which occurs in the empty auricle.

TABLE I.

Exp.	Weight of auricle in mg.	Oxygen used by normal auricle in c.mm. per hour		Concentration KCl employed	O_2 used by arrested auricle	
		Filled	Empty		c.mm. per hour	c.c. per g. per hour
116	19	32	—	0·16	7	0·37
124	15	11·3	4	0·16	7·6	0·51
132	10	11·0	—	0·16	7·0	0·7
135	10	6·6	—	0·10	2·7	0·27
150	9	9·1	—	0·10	3·8	0·42

The following is an example of an experiment conducted in oxygen.

Exp. 203. Weight of auricle 9 mg.

Condition of auricle	Filling in c.c.	Frequency per min.	Oxygen used in c.mm. per hour		Work equiv. of oxygen used (*b*) in g. cm. per beat	Potential energy released in g. cm. per beat
			(*a*) Total	(*b*) Minus basal consumption		
Serum-Ringer	0·04	47	24·5	16	1·2	0·34
Empty	0	40	8·5	—	—	—
0·05 p.c. KCl added	0·04	35	18·5	10	1·01	0·22
0·10 p.c. KCl added	0·04	0	7·5	—	—	—

This experiment shows that the oxygen consumption of the auricle arrested by potassium excess is similar to the oxygen consumption of the empty auricle. Moreover, an excess of potassium insufficient to arrest the heart causes a well-marked fall in oxygen consumption. Concentrations of potassium chloride less than 0·06 p.c. produced no certain alteration in either the potential energy released nor in the oxygen consumed.

Effect of acid.

Our serum-Ringer mixture had a *p*H of 7·5. The effect of acidity on the auricle was to reduce the frequency and at the same time to reduce both the mechanical response and the oxygen consumption. Within the

limits of error of our method the oxygen consumption and mechanical response appeared to be reduced equally as is shown by the following figures.

	pH 6·2	pH 6·0
Oxygen consumption in excess of basal as p.c. of normal	30	0
Work per hour as p.c. of normal	28	4

Effect of calcium lack.

Calcium lack was produced by adding sodium citrate to the serum-Ringer mixture. The quantities of fluid available were small and the intensity of action produced by any mixture depended partly on the number of times the auricle was washed out with the mixture. Two washings with a mixture containing 5 millimolar sodium citrate produced a considerable reduction in the response. The following are the results of a typical experiment.

Exp. 201. In oxygen throughout. Auricle weight 13 mg.

Fluid in heart	Filling in c.c.	Frequency per min.	Oxygen used in c.mm. per hour (*a*) Total	(*b*) Minus basal rate	Work equiv. of oxygen used (*b*) per beat in g. cm.	Potential energy released in g. cm. per beat
Serum-Ringer	0·08	45	22·5	13	1·02	0·50
Empty	0	—	9·5	—	—	—
Serum-Ringer + 5 m. molar sodium citrate	0·09	41	18·6	9·1	0·78	0·35
Serum-Ringer + 10 m. molar sodium citrate	0·09	40	8·5	0	—	0·1

The following results from four experiments show the relative effect of varying quantities of citrate on the work and oxygen consumption of the auricle. These experiments were conducted in air.

	(i)	(ii)	(iii)	(iv)	(v)	(vi)
Oxygen consumption due to contraction as p.c. of normal	80	55	32	29	18	15
Potential energy released as p.c. of normal	63	66	17	38	27	10

These figures show that lack of calcium reduces the oxygen consumed and the potential energy released by the heart approximately equally.

Effect of prolonged isolation on the auricle.

The frog's auricle has a remarkable survival power and if removed with aseptic precautions and perfused with serum or blood may survive and beat regularly for more than a week.

Most of our experiments were stopped by sepsis which usually appeared

after 48 hours and caused death of the heart with a rapid rise in oxygen consumption. Apart from this sepsis the oxygen consumption of the auricle remained remarkably steady provided the filling was kept constant. The basal metabolism did not fall but rather appeared to rise.

	Oxygen used by empty auricle in c.mm. per hour	
Exp.	Up to 12 hours after isolation	24 hours to 36 hours after isolation
165	3·12	5·77
164	2·98	4·73

Although the total oxygen use of the auricle did not decrease greatly after 24 hours' isolation, yet the potential energy released fell considerably, and hence the efficiency was greatly reduced as is shown by the following figures.

			Oxygen used in c.mm.		Work equiv. of oxygen used (*b*) in g. cm. per beat	Potential energy released in g. cm. per beat	P.c. efficiency
Exp.	Time in hours after isolation	Frequency per min.	(*a*) Total	(*b*) Minus basal consumption			
104	0–12	48	30	24	1·69	0·70	42
	24–36	40	20·3	14·3	1·26	0·24	17
147	0–12	38	22	17	1·58	0·64	40
	24–36	40	22·5	17·5	1·55	0·14	9

We have observed (2) a similar decline in efficiency in the case of the tortoise auricle.

The effect of Ringer's fluid on the auricle.

When Ringer's fluid was substituted for serum the oxygen consumption showed a rapid fall, similar to, but more rapid than, that observed in the case of the frog's ventricle (1). The replacement of Ringer's fluid by serum caused the oxygen consumption to rise again to its original level. These results are shown in the following experiment.

Exp. 174. Auricle suspended in air. Weight 12 mg. The fluid was changed hourly.

Time in hours	Fluid in cannula	Frequency per min.	Oxygen used in c.mm. per hour	Oxygen use due to contraction	Work equiv. of oxygen used in g. cm. per beat	Potential energy released in g. cm. per beat	P.c. efficiency
0–1	Ringer	30	9·5	5·4	0·64	—	—
1–2	,,	28	7·3	3·2	0·41	—	—
2–3	,,	24	7·4	3·3	0·49	0·28	57
3–4½	Serum	34	11·6	6·8	0·71	0·38	54
5–6	Empty	—	4·8	—	—	—	—
6–7	Washed Ringer's fluid and emptied	—	4·1	—	—	—	—

The figures show that washing with Ringer's fluid causes but little decrease in the resting metabolism of the auricle, although it causes a great decrease in the oxygen consumption due to the contraction process.

In a previous paper we expressed the opinion that perfusion with Ringer's fluid caused a greater decrease in the oxygen consumption than in the mechanical response of the heart. We have tested this in several experiments and have found that within the limits of error of our apparatus the substitution of Ringer's fluid for serum causes a reduction in the oxygen consumption (in excess of the basal metabolism) which is proportional to the reduction in the potential energy released by the auricle.

We found that the amplitude of the auxobaric response was a fallacious method of estimating the mechanical response of the auricle, for the change from serum to Ringer's fluid frequently produced no alteration in the auxobaric response and yet produced a considerable reduction in the isochoric response.

Discussion.

Our experiments on the action of ions, etc., on the frog's auricle confirm the conclusions drawn from experiments on the frog's ventricle.

In the experiments on the auricle certain possible sources of error present in our previous experiments have been eliminated. In the first place the measurements of the oxygen consumption of the frog's ventricle when arrested were of doubtful value because of the possibility that the tissue was suffering from oxygen lack. This source of error has been eliminated by using the auricle which is a much thinner tissue than the ventricle and also by keeping the tissue in an atmosphere of oxygen instead of in air.

The mechanical response of the frog's ventricle was estimated by measuring the added pressure at a given volume, whereas with the auricle the potential energy released per beat was estimated. The latter method is more accurate than the former, but a study of the results shows that variations in the pressure added when the diastolic filling is adequate gives a fairly accurate indication of the variations in the potential energy released.

The frog's auricle is such a delicate tissue that it is only possible to get approximate estimations of the work performed when partial paralysis is produced by drugs. Prolonged isolation in the presence of serum produces, however, a condition in which the oxygen consumption is but little reduced whilst there is a great reduction in the mechanical response, and in consequence a very obvious decrease in efficiency. It is therefore possible to reduce the mechanical response of the heart without reducing the

oxygen consumption. On the other hand, the oxygen consumption can be abolished by cyanide without affecting the mechanical response.

The ionic changes we have studied, and also ethyl urethane, appear, however, to affect the two factors, oxygen consumption and mechanical response, approximately equally. These facts can be explained on the hypothesis that excitation causes a primary chemical change, the products of which act on some structure in the muscle to produce the mechanical response and are then oxidised by a recovery process. The ionic changes studied by us, and ethyl urethane, appear to inhibit the primary chemical change and thus affect the mechanical response and the oxygen consumption equally. It is generally agreed that cyanide inhibits the recovery process. Prolonged isolation in serum, on the other hand, appears to injure the structure on which the products of the chemical change act, and hence causes a much greater reduction in the mechanical response than in the oxygen consumption.

Conclusions.

1. Within the limits of experimental error we have found that in the frog's auricle the oxygen consumption associated with the excitation process and the potential energy released are reduced in equal proportions by the following agencies, (*a*) ethyl urethane, (*b*) excess of potassium, (*c*) acid, (*d*) lack of calcium.

These results confirm conclusions which we have published previously.

2. The substitution of Ringer's fluid for a mixture of serum and Ringer's fluid also results in an equivalent reduction in oxygen use and potential energy released. Previously we expressed the opinion that these two factors might vary independently in this case.

3. A considerable oxygen consumption persists when the auricle is arrested by the agents mentioned above. This oxygen consumption is similar to or slightly less than the oxygen consumption of the empty auricle.

4. Prolonged isolation in presence of serum produces a condition in which the mechanical response is reduced much more than is the oxygen consumption, and hence there is a marked loss of efficiency.

The expenses of this research were defrayed by a grant from the Moray Fund of Edinburgh University.

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THE EFFECT OF VARIATIONS IN THE DIASTOLIC FILLING OF THE FROG'S VENTRICLE UPON THE DURATIONS OF THE REFRACTORY PERIOD AND THE MECHANICAL RESPONSE.

By T. M. TSO.

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The experiments described below were undertaken to determine whether the durations of the refractory period and of the mechanical response of the isolated frog's heart were altered by variations in the diastolic filling. Such variations have been shown to influence profoundly the pressure added by contraction (1,2,3,4), the oxygen consumption (5,6), and the heat liberated by the heart (7). In particular, Clark and White (6) have shown that the empty contracting heart uses very little more oxygen than does the empty arrested heart, whereas the heart contracting when filled uses four or five times this quantity. The amount of chemical change induced by excitation can therefore be varied very extensively by simply varying the diastolic filling of the heart.

The purpose of the experiments described below was to determine whether such variations in the amount of potential energy released by excitation were associated with any corresponding change in the duration of either the refractory period or of the mechanical response.

Methods.

The isolated ventricle of *R. temporaria* was used and the experiments were carried out in winter and spring at room temperature. The temperature varied on different days from 12° C. to 15° C. but remained constant during the course of any experiment. The Ringer's fluid used had the percentage composition of NaCl 0·65; KCl 0·015; $CaCl_2$ (anhyd.) 0·011; Na_2HPO_4 and NaH_2PO_4 0·05. The phosphate was added from a stock phosphate solution with a *p*H of 7·5.

The apparatus for perfusing the ventricle, for varying its diastolic filling, and for recording its isochoric response was similar to that described by Clark (8). The heart contracted under isobaric conditions

except when measurements were taken, and then isochoric conditions were maintained.

A Lewis rotary contact breaker provided with two pairs of cams was used to determine the refractory period. Each pair of cams was connected with an induction coil, and the wiring was arranged as shown in Fig. 1.

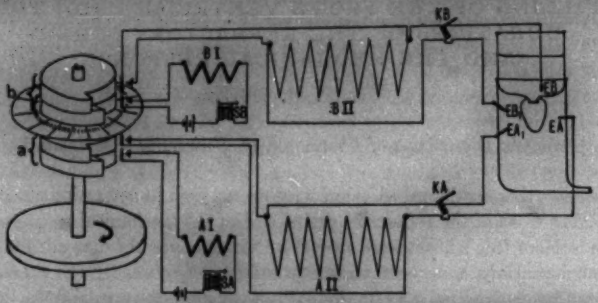

Fig. 1. Arrangement for providing pairs of break shocks with varying frequency and intervals. *A I* and *A II*. Primary and secondary coils respectively of induction coil *A*. *B I* and *B II*. Primary and secondary coils respectively of induction coil *B*. *SA* and *SB*. Signal markers. *KA* and *KB*. Short-circuiting keys. *EA* and EA_1. Electrodes of circuit *A* (initial stimulus). *EB* and EB_1. Electrodes of circuit *B* (second stimulus). *a* and *b*. Contact makers for circuits *A* and *B*.

The make shock was short-circuited and only the break shock reached the preparation.

One coil (*A*) and one pair of cams (*a*) were used to produce the initial stimulus, and the fundamental rate of stimulation was usually 18 per minute. This frequency could be varied by varying the rate of rotation of the contact breaker. The second coil (*B*) and the second pair of cams (*b*) provided the second or extra stimulus. The distance between cams *a* and *b* could be varied, and thus the second stimulus could be thrown in at any point in the cardiac cycle. Two signal markers recorded the stimuli on the tracing and the duration of the interval between the stimuli was measured from these records.

The strengths of the stimuli were adjusted by moving the secondary coils, and these were calibrated with a microammeter. The strength of stimulus *A* was always above the threshold strength, being usually three times that of the threshold strength. The strength of stimulus *B* was varied according to circumstances.

The determination of the refractory period.

The absolute refractory period (A.R.P.) was determined by the usual method of sending in a pair of stimuli and finding out the earliest point in the cardiac cycle at which the second stimulus (*i.e.* stimulus *B*) was capable of producing a response. For this determination the strength of stimulus *B* was made about 40 times the threshold strength. In all cases five pairs of stimuli were passed in succession, and the result was considered positive, if a second contraction was obtained after any or all of these paired stimuli. The duration of the refractory period decreases when the frequency of stimulation is increased, and hence once the second stimulus had produced a contraction, it continued to do so. The practice of observing the results of five successive pairs of stimuli was a safeguard against errors which will be considered later.

In determining the relative refractory period (R.R.P.) the minimal effective strength of the second stimulus at various intervals was determined and a curve of recovery of excitability was thus plotted. The perfusion fluid used was on the alkaline side of neutrality (*p*H 7·5) and a supernormal phase was not usually present. Therefore, the curve of recovery of excitability was usually measured only as far as the end of the relative refractory period.

The tracings obtained were measured with a reading microscope. In some cases tracings were made in smoked celluloid films, and the image was projected on to a screen where it was measured. The results obtained by the two methods were in close agreement. The tracings could be measured accurately to the nearest 0·05″; hence the error in measurement was about 3 p.c. of the duration of the cardiac cycle.

Sources of error.

(*a*) *Short-circuiting through electrodes.* It was found that the arrangement of the electrodes was a matter of great importance. At first the electrodes were arranged as in Fig. 2. This arrangement had to be discarded because when the key (*KB*) in circuit *B* was closed the current in circuit *A* instead of passing from *EA* to EA_1 (through the ventricle) often passed by the alternative route EA–EB–EB_1–EA_1, and hence failed to stimulate the heart. Similarly, the current in circuit *B* passed by a corresponding alternative route when the key (*KA*) in circuit *A* was closed. The arrangement of electrodes shown in Fig. 1 was designed to overcome this difficulty.

Under this arrangement there was only one way for a current in

circuit B to complete its course, namely through the ventricle (EB–EB_1). This ensured uniformity in the strength of stimulus B irrespective of the

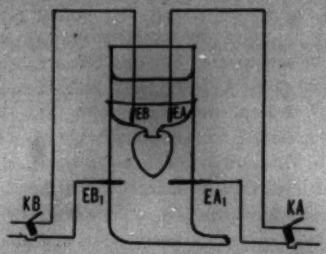

Fig. 2. Original arrangement of electrodes. Letters as in Fig. 1.

opening or closing of the key in circuit A. As to stimulus A, the alternative route (EA_1–EB_1–EB–EA) always offered greater resistance than did the direct route (EA–EA_1) no matter whether the key in circuit B was open or closed. Moreover, variations in the strength of the initial stimulus (circuit A) were less important than variations in the strength of the second stimulus (circuit B), for the initial stimulus was well above threshold strength. The question as to whether variations in the strength of the primary stimulus affect the duration of the refractory period has been a matter of controversy, but the most recent evidence (Hermann and Umrath(9)) shows that they do not do so.

(*b*) *Inadequate perfusion.* Boesch(10) showed that lack of oxygen shortened the refractory period of the frog's heart. Inadequate perfusion will therefore probably shorten the refractory period. To avoid this error isochoric conditions were maintained only for six or eight successive contractions, and then isobaric conditions were substituted and the supply of oxygenated Ringer's fluid restored.

(*c*) *Hypodynamic condition.* Prolonged washing of the frog's heart with Ringer's fluid produces a hypodynamic state, as has been shown by Clark(11). Junkmann(12) showed that the refractory period of the frog's heart shortened considerably after prolonged perfusion. This fact was confirmed by the writer. The curve of recovery of excitability of a heart before and after two hours' perfusion with Ringer's fluid is shown in Fig. 3. This shows a shortening of both the A.R.P. and the R.R.P. This fact made it necessary to do frequent controls when experiments of long duration were made.

(*d*) *Spontaneous contractions.* Ventricles sometimes commenced spontaneous contractions after one or two hours' perfusion. In such cases it was usually necessary to stop the experiment.

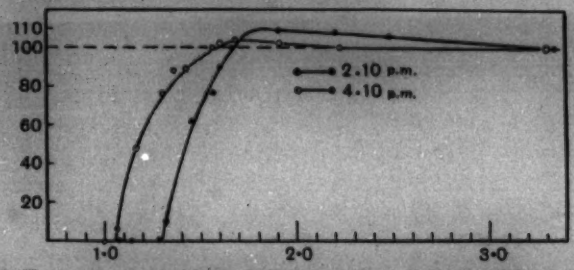

Fig. 3. The recovery of excitability in the frog's ventricle. Ordinate: excitability expressed as percentage of resting value. Abscissa: time in seconds.
Exp. 9. iii. 29. Filling 0·06 c.c. Interval of 2 hours between the two curves.

Effect of diastolic filling on the refractory period.

The development of the hypodynamic condition produced a considerable shortening of the refractory period and therefore it was important to perform any series of comparative determinations as quickly as possible. For this reason in most experiments only the commencement of

Fig. 4. The refractory period and mechanical response with fillings of 0·05 and 0·01 c.c. From below upwards the tracings show: time marker in 0·5 seconds; initial stimulus; isochoric response of ventricle; second stimulus. The points corresponding in time are marked at the commencement of the tracings. (Downstroke = systole. Tracing reads from left to right.)

the A.R.P. and the end of the R.R.P. were determined. Typical results are shown in Table I, and Figs. 4 and 5 show specimens of the tracings obtained. The figures in Table I show that variations in diastolic filling,

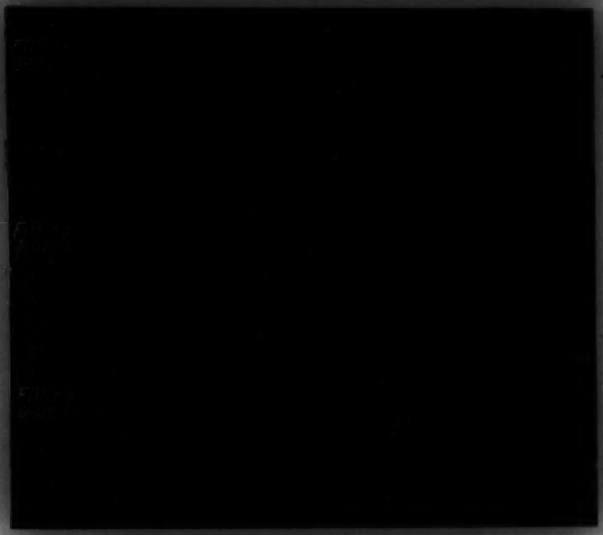

Fig. 5. The refractory period of the frog's ventricle with varied fillings.

Three tracings are shown with fillings of 0·01, 0·02 and 0·03 c.c. respectively. Each tracing, from below upwards, shows: initial stimulus; time marker 0·5 seconds; second stimulus; isochoric response. In each of three tracings the interval between the first and second stimulus in the first half of the tracing is 20 divisions or 0·95 seconds, and in the second half of the tracing 21 divisions or 1·05 seconds. The strength of the stimuli was kept constant throughout. In all three tracings the second stimulus did not produce a response at an interval of 0·95 seconds but produced a response at an interval of 1·05 seconds. Therefore no measurable alteration in the refractory period was produced by the variation in filling.

sufficient to alter the added pressure sixfold, do not produce any measurable change in the duration of the refractory period. In some experiments the curve of recovery of excitation was plotted with different fillings. Such curves are given in Fig. 6 and show that changes in diastolic filling do not affect the rate of recovery of excitability.

TABLE I.

Exp. 27. xi. 28. Frequency 24 contractions per minute.

Filling in c.c.	A.S.P. in seconds	End of R.S.P. in seconds	Pressure added by contraction in cm. H_2O
0·01	<1·00>0·95	1·39	10
0·03	<1·02>0·95	1·41	50
0·06	<1·00>0·97	1·37	62
0·07	<1·01>0·98	1·39	64

Exp. 21. ii. 29. Frequency 18 contractions per minute.

0·01	<1·24>1·18	1·79	11
0·03	<1·23>1·18	1·76	37
0·05	<1·23>1·19	1·78	58

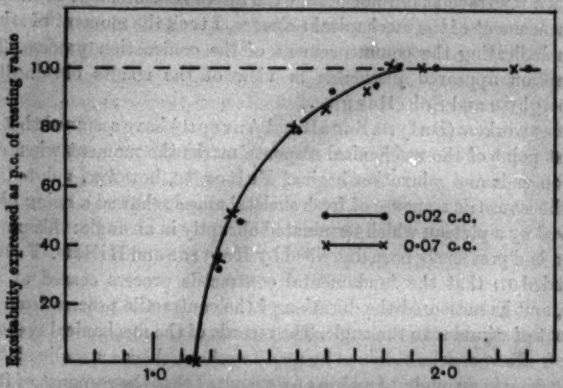

Fig. 6. Recovery of excitability in the frog's ventricle with 0·02 and 0·07 c.c. filling. Ordinate and abscissa as in Fig. 3.

The measurement of the duration of the mechanical response.

There is a certain difficulty in deciding what points should be taken as representing the commencement and end of the mechanical response. The usual methods of recording the mechanical response of a heart to a stimulus show a considerable latent period. All workers who have used specially delicate methods for recording agree that the greater part of the latent period, as shown with ordinary apparatus, is due to mechanical lag. De Jongh(13) actually concluded that the mechanical and electrical responses appeared simultaneously. Samojloff(14) found that the latent period of the electrical response of the frog's ventricle when a strong stimulus was used was about 0·024 seconds, but Schellong(15) found

that this latent period, in the case of strips of frog's ventricle, was only 0·002 seconds or less.

My recording apparatus was designed to measure the pressures produced by the mechanical response, and was not sufficiently delicate to measure the true latent period. Examination of the curves in Fig. 8 shows that the rise of pressure follows an S-shaped curve, the commencement of which cannot be determined accurately. In this figure the interval between the stimulus and the steep curve varies from 0·36 to about 0·2 seconds, but the interval between the stimulus and the commencement of the gradual rise is less than half these amounts.

Since my tracings did not permit the direct measurement of the true commencement of the mechanical response, I took the moment of stimulus as indicating the commencement of the contraction process. This assumption appeared justifiable in view of the results obtained by de Jongh(13) and Schellong(15).

Some workers (Daly(3), Segall and Anrep(4)) have assumed that the highest point of the mechanical response marks the moment when contraction ends and relaxation begins. Fulton(16), however, pointed out that the isometric response of fresh skeletal muscle showed a rising phase followed by a plateau which terminated abruptly in an angle: this phenomenon had previously been described by Hartree and Hill(23). Fulton concluded(17) that the fundamental contractile process ceased at this angle, and he measured the duration of the contractile process from the moment of stimulus to the angle. The records of the mechanical response of the frog's ventricle, obtained by many workers, show a systolic plateau terminating in an angle. Junkmann(12) noted that the response of fresh ventricles showed this plateau, but that the plateau changed to a hump after prolonged perfusion.

My records confirm this. Fig. 7 shows the typical plateau and angle

Fig. 7. Isochoric response of fresh ventricle, showing well-marked "angle." Time, etc., as in Fig. 4.

obtained with a fresh ventricle, whilst Fig. 4 shows the hump-shaped response obtained after one or two hours' perfusion.

This angle indicates a change in the state of the muscle much more clearly than does the highest point of the response, and therefore I measured the contractile process as commencing at the moment of stimulus and terminating at the angle.

This measurement was, however, made from the moment of an electrical stimulus up to the appearance of a change in the mechanical response, and therefore any instrumental lag would form a serious source of error. I tested directly the time taken for pressure applied at the ventricle to be recorded by the manometer lever and found that this was not more than 0·01 seconds. The exaggerated latent period is therefore not due to instrumental lag, but is due to a delay between the onset of contraction in the ventricle and the production of pressure in its contents. The fact that the rise of pressure follows an S-shaped curve supports the view that the onset of contraction in the ventricle does not immediately produce a rise of pressure. On the other hand, the records show a sharp fall at the onset of relaxation, and this sharp fall probably gives a true measure of the moment of relaxation.

The influence of diastolic filling on the duration of the mechanical response.

This question has been investigated by a number of workers, especially Daly(3) and Segall and Anrep(4). Their results in general agreed with each other, and according to Segall and Anrep, the total duration, the period of rising tension, and the period of falling tension were prolonged by increased diastolic filling. They further observed that, for the period of rising tension, the increase was more marked with smaller fillings, reaching a maximum when the heart was filled to two-thirds of its full capacity; while for the period of falling tension, the maximum increase occurred with the larger fillings. These workers divided the duration of contraction into the period of rising tension and the period of falling tension. The former was measured from the point where the curve left the base line to the middle of the flattened top, and the latter from this point to where the curve returned once more to the base line. For the reasons already mentioned the writer measured the duration of the contractile process from the stimulus to the angle. The relaxation process was measured from the angle to the point where 90 p.c. of relaxation had occurred. The lever performed a circular movement and a correction was made for this error.

Table II and Fig. 8 show the effect of variations in diastolic filling on the duration of the contractile process and relaxation. These results show that the duration of the period from the stimulus to the angle actually decreases slightly with increase of filling. The decrease observed (0·06

TABLE II.

Filling in c.c.	Stimulus to "angle" or D.M.B. in seconds	"Latent period" in seconds	D.M.B. minus "L.P." in seconds	"Angle" to 9/10 relaxation in seconds	Pressure added by contraction in cm. of H_2O
Exp. 22. i. 29. Frequency 9 contractions per minute.					
0·01	2·00	0·36	1·64	0·75	39
0·02	1·97	0·29	1·68	0·88	63
0·03	1·95	0·20	1·75	0·90	85
0·05	1·94	0·16	1·78	1·37	93
0·06	1·94	0·06	1·88	1·37	92
Exp. 9. ii. 29. Frequency 10 contractions per minute.					
0·01	1·73	0·20	1·53	0·62	35
0·02	1·73	0·16	1·57	0·71	52
0·03	1·68	0·14	1·54	0·85	64
0·04	1·66	0·12	1·54	0·92	74
0·05	1·70	0·11	1·59	0·97	78
0·06	1·71	0·11	1·60	1·10	83
0·08	1·67	0·07	1·60	1·30	85

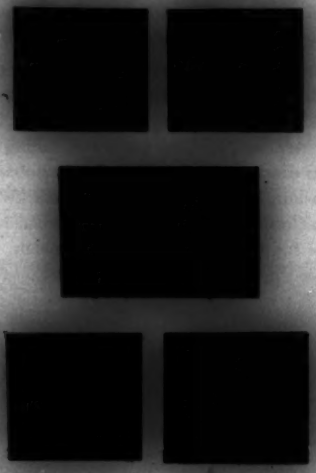

Fig. 8. Tracings showing duration and height of mechanical response of ventricle with fillings varying from 0·01 to 0·06 c.c. (Figures shown in Table II. Exp. 22. i. 29.)

seconds) is however very close to the possible error in measurement. On the other hand, the relaxation process is greatly prolonged by increased filling. The first result is opposed to, whilst the second result is in agreement with, the results of Segall and Anrep.

This divergence in results is due to the different methods employed in measurement. My method of measurement of the duration of the contractile process included the apparent latent period, and this period is greater with small fillings than with large fillings; if this period be subtracted from my measurements, they then show an apparent increase in the duration of the contractile process when the filling is increased (cf. Table II, column 4), and thus agree with the results of previous workers.

As regards skeletal muscle Hartree and Hill(23) showed that increase of extension of the frog's sartorius had little or no effect upon the duration of the rising phase of the mechanical response but a large effect in slowing the relaxation. Fulton(18) found that increase in initial tension in the frog's gastrocnemius caused an increase in the duration of the contractile process measured from stimulus to angle. His figures show, however, that increase in initial tension produced a far greater effect on the added tension than on the duration of the contractile process. For instance, in one of his experiments the duration only increased 13 p.c. when the added tension increased 80 p.c.

The relation between the absolute refractory period and the duration of the contractile process.

My results, as summarized in Table III, show that with moderate fillings, the A.R.P. ends about 0·1 seconds before the angle, which was taken

TABLE III.

Date of Exp.	Frequency	D.M.R. in seconds	A.R.P.* in seconds	Difference between D.M.R. and A.R.P.
16. iii. 29	18	1·48	1·32	0·16
5. iii. 29	18	0·95	0·86	0·09
19. iii. 29	18	1·40	1·33	0·07
19. iii. 29	10	1·56	1·44	0·12
19. iii. 29	28	1·18	1·04	0·14
14. iii. 29	18	1·35	1·24	0·11
18. iii. 29	18	1·17	1·05	0·12
18. iii. 29	10	1·29	1·18	0·11
18. ii. 29	27	1·04	0·91	0·13

* The A.R.P. given here is the average of the longest time interval between stimuli which failed to yield a positive result and the shortest interval which did yield a positive result.

to indicate the end of the contractile process. This result agrees with the findings of previous workers (Trendelenburg(19), Junkmann(12),

Holzlöhner(20)). The figures in Table III also show that the absolute duration of these periods varies considerably in different hearts, but that the relation between the two periods remains fairly constant.

Dennig(21) showed that various influences such as excess of calcium or excess of potassium could cause extensive and uncorrelated changes in the duration of the mechanical response and in the A.R.P. Therefore the fact that increase in filling does not alter the A.R.P. but causes a slight shortening of the duration of the contractile process does not appear to be of much significance.

Discussion.

My results show that the recovery of excitability occurs at the same rate in a frog's ventricle that is almost empty as in one that has an adequate diastolic filling. The results also show that increase in diastolic filling causes a slight decrease in the duration of the contractile process, although it greatly prolongs the process of relaxation.

The results of other workers show that increase in diastolic filling, and consequent increase in the length of muscle fibres, cause in cardiac, as in skeletal, muscle a great increase in the amount of chemical change produced by excitation. My results show therefore that an increase in the amount of chemical change produced by excitation of the frog's ventricle does not necessarily prolong the duration of either the contractile process or the refractory period.

Adrian(22) has produced evidence in favour of the view that the loss of excitability that follows excitation is due to a change of permeability in some semi-permeable surface in the muscle cell, and that the duration of the refractory period measures the period required to restore the normal condition of semi-permeability.

The mechanical response is caused by chemical changes induced by the change in permeability, and the magnitude of these chemical changes is conditioned chiefly by the initial length of the muscle fibre. My experiments show that in the frog's ventricle variations in the initial length of the fibres, and consequent variations in the extent of the chemical change, do not affect the rate at which the excitability is restored to normal, and produce little effect on the duration of the contractile process.

Conclusions.

1. Variations in the diastolic filling of the isolated frog's ventricle do not result in any measurable alteration in the duration of either the absolute or the relative refractory period.

2. The duration of mechanical response from the moment of stimulus to the end of the systolic plateau is not increased by increasing the diastolic filling of the ventricle but is either unchanged or else slightly decreased.

3. Under the conditions studied the end of the absolute refractory period preceded the end of the systolic plateau by about 0·1 second.

I wish to express my sincere thanks to Professor A. J. Clark for suggesting this work and for his help and guidance throughout its execution.

The expenses of the research were defrayed by a grant from the Earl of Moray Research Fund of Edinburgh University.

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PROCEEDINGS
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Heart block in rice-fed pigeons. By C. W. Carter and A. N. Drury.
(*Biochemical Laboratory, Oxford, and Pathological Laboratory, Cambridge.*)

Pathological lesions have been described by Vedder and Clark(1), McCarrison(2) and others in the heart, central nervous system and peripheral nerves of fowls and pigeons fed on a polished rice diet. Such pigeons were found by Peters to exhibit an abnormal cardiac rhythm. We have found this to be due to heart block. Over 100 cases of this condition have been observed, the severity of the block ranging from an occasional missed ventricular beat to complete dissociation of rhythm of auricles and ventricles. The heart block is established at an earlier date after the commencement of a "B" deficient diet than the polyneuritis and head retraction. Signs of block usually appear within 10 to 15 days, whereas head retraction is generally exhibited between the 20th and 30th day. In a few cases electrocardiographic records have been obtained, and two typical examples are tabulated below. The birds were anæsthetized with ether during the experiment and the electrical records taken at short intervals over a period of about half an hour.

Bird	Days on polished rice	Aur. rate (per min.)	Vent. rate (per min.)	*P–R* int. (in sec.)
1	20	225	200	(Occas. dropped beats)
		136	136	0·13
		156	100	(Occas. dropped beats)
		155	155	0·18
		140	140	0·17
		Right vagus nerve sectioned.		
		225	225	0·12
		232	200	(Occas. dropped beats)
		240	210	(Occas. dropped beats)
		Left vagus nerve sectioned.		
		381	381	0·08
		390	390	0·08
		325	325	0·08
		300	300	0·08
2	20	466	466*	0·08
		400	230†	(2 : 1, 1 : 1)
		325	180	(2 : 1, 1 : 1)
		366	118	(4 : 1, 3 : 1)
		Atropine sulphate 0·003 gm. injected intra-muscularly.		
		350	350	0·09
		340	340	0·08
		370	370	0·08
		323	323	0·08

* Immediately after exercise.
† Resting.

In both these examples it will be observed that there is a partial degree of heart block and also considerable variation in the auricular rate, both of which vary from minute to minute. The electrical records show that the *P*-waves and ventricular complexes have normal contours. Section of both vagi or paralysis of vagal tone with atropine completely abolishes the block; the electrical records at this stage resembling the records from a normal pigeon in every respect. In a number of birds which have been allowed to recover from the operation of vagal section it has been found that the heart block is permanently abolished. An attempt has been made to discover whether the cardiac condition is to be attributed to the influence of a normal vagal tone upon a hyper-sensitive heart, or whether it is of central origin. The condition is not due to increased intra-cranial pressure from cerebral œdema. The cerebrum was removed in one bird through a large opening made in the skull and the bird, after recovery from the operation, was placed on a diet of polished rice. In 16 days well-marked heart block was established. In another set of experiments both vagi were sectioned in a rice-fed and a normal control bird, and 0·016 gm. pilocarpine nitrate was injected intramuscularly. The results are tabulated below.

	Min.	Normal Vent. rate per min.	Rice-fed Vent. rate per min.
Before vagal section	—	185	100
After vagal section	—	368	400
After pilocarpine nitrate, 0·016 gm.	1	240	232
" " "	5	224	232
" " "	10	240	256

As this dose of pilocarpine produced a similar degree of vagus stimulation in the two birds, it is difficult to consider that the heart of the rice-fed bird is in a hyper-sensitive condition. It is thought that the heart block observed in pigeons fed on a diet of polished rice is the result of an over-action of the vagal centres.

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Observations upon the oxygen consumption of "colpidium colpoda." By RUDOLPH A. PETERS.

These observations form part of a research into the nutritive requirements of protozoa. Growing cultures of *Colpidium colpoda* use large amounts of oxygen, approximately 0·200 c.c. per million organisms in the first week of the culture. Older cultures take up less oxygen, the lowest

figure obtained being 0·050 c.c. per million. Taking a maximum length for the organism of 60μ and a breadth of 20μ, which are outside figures, and reckoning the organism as a cylinder, the oxygen consumption works out as ten litres per kilo per hour approximately. This is probably a low estimate. Moderate amounts of KCN *M*/500 do not affect the oxygen uptake. Larger amounts, *M*/200 KCN, lead to a progressive fall in oxygen uptake, accompanied with cytolysis of the organisms. Heating to 40° C. for 15 minutes kills the organisms and stops oxygen uptake.

Quantitative observations have been made previously by Vernon(1) upon *Collozoum*, and by Wachendorff(2) upon *Colpidium colpoda*, both of whom obtained high values for the oxygen consumption. The latter obtained values varying from 0·065–0·386 c.c. oxygen per million organisms, an amount very similar to the figures for my strain. Their observations were not made upon growing cultures free from bacteria. The cultures used in the above experiments are believed to be free from bacteria. They have been obtained from the original strain isolated by me in 1919(3), and grown upon the synthetic medium previously described with additions of prepared sterilized yeast or of glaxo, without which additions it has been impossible to make them grow. This fact, taken with the finding that respiration in the cultures is completely stopped by a short heating at 40° C., supports the view that they are not contaminated. Contrary to my previous assertions(4), therefore, it seems that growth factors must be supplied to these organisms, and that these must have been elaborated in the original cultures by unrecognized bacteria(5). This view has been put forward by Oehler(6), and by Cutler and Crump(7). Up to the present time I have been unable to make continuous subcultures of the strain used in the present experiments in the absence of solid matter in the medium.

(I am much indebted to Dr Walker, to Mr Carter, and also to Mr Wakelin for their kind assistance at various times during the last eight years in keeping alive the original strain. This has also been used by both Dr Cutler and Dr Crowther in their experiments. I also wish to thank Dr Carleton for his willing histological assistance.)

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Physiological action of X-rays. By SAMSON WRIGHT and H. A. BULMAN. (*Middlesex Hospital.*) (*Preliminary Communication.*)

Radiation employed. The X-rays were of a very penetrating character; exposure to these rays at the distance employed (57 cm.) kills the cells of Jensen's rat sarcoma *in vitro* in 150 min.

General Results. Irradiation of entire body. (i) *Cat*: irradiation for 75 min. or over, death invariably results in 5 to 12 days; 50 to 60 min., a proportion of the animals survive; 30 min., recovery always takes place. (ii) *Rabbit*: considerably greater resistance is displayed; irradiation for 100 min. or less, recovery always occurs; death may result from exposure of 145 min. duration.

In the cat the principal symptom is loss of appetite which in severe cases may be complete; no food or drink may be taken for as long as 12 days; 40 p.c. of the initial weight may be lost and considerable general weakness is present. Recovery may however occur after a period of inanition of this duration; life is not definitely prolonged by daily repeated injection of saline subcutaneously, with or without adrenaline.

Blood Changes. Cat. 1. *Fatal Cases.* (*a*) The total white count (normal range 9000–30,000) after an initial transient and inconstant increase, decreases fairly steadily; after 3 to 5 days the count may be reduced to a few hundreds or less per c.cm.; the animal may survive with this state of aleukia for 3 to 9 days. (i) The lymphocytes begin to decrease within a few hours and disappear almost completely on 2nd to 3rd day. (ii) The polymorphs may show an initial increase, then decline more gradually than the lymphocytes; on the 3rd to 5th day when total count is very low they may constitute 95 p.c. of the cells present.

(*b*) *Red Count* (range 5 to 13 millions). The immediate effect is variable: irregular fluctuations, or a rise or a mild decline; there is crenation, poikilocytosis, anisocytosis, appearance of numerous microcytes and signs of intravascular fragmentation. In a proportion of the cases a severe anæmia develops acutely several days before death accompanied by evidence of hæmolysis (positive Fouchet test and positive indirect van den Bergh's test for bilirubin).

(*c*) *Platelets* (range from under 0·5 to 5 million). No decrease has been noted in fatal cases even when there was evidence of infection.

2. *Non-fatal cases.* (*a*) The white cells show the same general changes, but with smaller doses the decrease in the polymorphs is less marked; about the 9th day recovery sets in, and is complete in about 1 to 2 months.

(*b*) Anæmia becomes definite after 10 to 15 days; the red count remains low for about 2 weeks, then slow recovery sets in which is complete in about 2 months.

Rabbit. In fatal cases the white cell changes generally resemble those in the cat, but the decline in the polymorphs is not so marked; anæmia may appear just before the end.

In the non-fatal cases anæmia is not a marked feature.

Rôle of infection. Correlated with the changes in the white cells is the fact that in many of the fatal cases evidence of infection was present—*e.g.* gastro-enteritis, pneumonia, abscesses of lung, empyema, pericarditis, and otitis media; even when no localized infection was found blood culture sometimes proved positive.

Comparison with chronic histamine poisoning. A clinical picture somewhat resembling X-ray sickness can be produced in the cat by subcutaneous injection of 24 mg. histamine acid-phosphate several times daily for a number of days. The blood changes however are strikingly dissimilar—such doses of histamine produce a marked rise in the white count, to 40,000 or even 55,000 per c.cm., due as a rule to an increase in the number of polymorphs without notable change in the total number of lymphocytes; the polymorphs may constitute over 90 p.c. of the total white cells.

Irradiation of chest only. (Cat.) It is assumed that the lungs, heart and thoracic parietes contain about one-eighth of the total blood volume. The chest was irradiated (with the rest of the body screened) for 260, 405 and 490 min., corresponding to an exposure of the whole blood-stream for 30, 50 and 60 min. respectively; in this way one can determine to what extent the blood changes described above are due to a direct action on the blood and how much is due to an action on the blood-forming tissues. In such experiments when death results it seems to be due to direct injury to the thoracic viscera. The blood changes are as follows: no anæmia develops; the platelet count is unaffected; the decline in the lymphocytes is rapid and marked, but the decrease in the polymorphs is less evident. The white count recovers to normal after 10 to 14 days. It seems that the anæmia, decreased platelet count and persistent low white count, which are seen when the animal survives (for a sufficient time) radiation of the entire body, are due mainly to injury to the blood-forming organs.

Irradiation of abdomen alone. (Cat.) For 150 min. kills in about 5 days; the animals survive abdominal radiation for 75 min.

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October 19, 1929.

Effects of breathing oxygen at high pressures upon tissue gas tensions. By J. ARGYLL CAMPBELL.

Rabbits were enclosed within a small compression chamber, the pressure of which was raised by connecting up with a cylinder of gas under high pressure. The CO_2 produced was absorbed by soda-lime. Samples of gas were taken from a tube leading from the chamber. Tissue CO_2- and O_2-tensions were estimated by the method of injection of nitrogen under the skin and into the abdominal cavity (1). To obtain samples from the animal, to show the effects of the experiment, the chamber was opened immediately after rapid decompression. For control, experiments were carried out under exactly similar conditions except that the chamber was filled with air instead of almost pure oxygen. In some cases figures were obtained for the same animals under the two conditions; in others different animals are compared in Table I. With high pressures of oxygen (average

TABLE I. Effects of breathing O_2 at high pressures.

Rabbit No.		O_2 pressure in air breathed		Bar. press. atmos.	Duration hr.	Tissue gas tensions mm. Hg							
						CO_2				O_2			
						Skin		Abd. cav.		Skin		Abd. cav.	
Air exps.	O_2 exps.	Air exps.	O_2 exps.			Air exps.	O_2 exps.	Air exps.	O_2 exps.	Air exps.	O_2 exps.	Air exps.	O_2 exps.
57	57	470	2055	+2·0	2·2	79	102	67	74	36	150	85	358
62	62	312	1746	+1·6	2·7	81	81	79	84	45	148	70	326
56	56	462	1340	+1·0	5·5	64	74	56	68	36	84	89	280
56	23 (D)*	680	3566	+4·0	1·5	92	77	66	95	92	213	212	430
56	57 (D)	680	3420	+4·0	1·5	92	121	66	213	92	170	212	3078
62	62 (D)	710	3625	+4·0	1·5	89	128	107	284	149	193	163	2910
62	63 (D)	710	3325	+4·0	1·5	89	214	107	274	149	190	163	483
57	55 (D)	470	2036	+2·0	2·2	79	77	67	213	36	325	85	306
Averages		562	2639	+2·8	2·3	83	109	77	163	79	184	135	1021

* (D) = died.

= 2639 mm. Hg or 3½ atmospheres) both the O_2- and CO_2-tensions in the tissues were markedly increased (average over 100 p.c.) as compared with the air experiments when the oxygen pressure was only about 562 mm. Hg or ¾ atmosphere on an average. It takes time for constancy to become established in such experiments and in most cases this was too short, but the figures obtained for the abdominal cavity indicate that the tissue

tensions must have been at least higher. In all the animals which eventually died the CO_2-tension in the abdominal cavity was distinctly higher than that for the survivors; in four out of five animals the CO_2-tension was 213 mm. Hg (30 p.c.) or more. This is the dangerous limit for rabbits. In Table II results are given for rabbits breathing about 30 p.c. of CO_2

TABLE II. Effects of breathing 27–30 p.c. CO_2 under ordinary barometric pressure.

				Tissue gas tensions mm. Hg							
				CO_2				O_2			
	Gas breathed in high CO_2 exp. mm. Hg			Skin		Abd. cav.		Skin		Abd. cav.	
Rabbit No.	CO_2	O_2	Duration hr.	In air	In CO_2	In air	In CO_2	In air	In CO_2	In air	In CO_2
65	188	228	2	44	123	45	159	20	21	31	42
64	220	224	2	50	166	49	192	17	14	36	35
66	191	214	2	50	171	49	201	31	22	35	36
22 (D)*	287	220	3	42	130	47	210	31	22	42	33
Averages	221	221	2·2	46	148	47	191	25	20	36	37

* (D) = died.

at ordinary barometric pressure; such a tension was sufficient to narcotize and even cause death. We have some evidence then that breathing O_2 at high pressure increases the tissue CO_2-tension to a dangerous level and this may be the cause of narcosis and convulsions produced by O_2 at high pressures. That is, O_2 poisoning at high pressures is concerned with CO_2. The venous blood must be arterial so far as its O_2 content is concerned; therefore, in accordance with the curves of Christiansen, Douglas and Haldane(2), the CO_2 is removed with difficulty from the tissues. Again, there is evidence that high pressure of O_2 causes constriction of blood vessels of the brain(3).

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The part played by venous obstruction in the development of a collateral circulation. By W. R. SPURRELL.

From the Physiological Laboratory, Guy's Hospital.

It has been shown, both clinically and experimentally, that the risk of gangrene in a limb, deprived of its main arterial supply, is considerably diminished if the main vein is tied. To study the part played by this venous obstruction, a collateral circulation was established in a series of animals with and without concomitant venous ligature and the resulting vascular response was examined.

Under ether fifteen cats were operated on; in each animal the right femoral artery and vein and the left femoral artery only were tied just below the origin of the circumflex vessels. In from two to twenty-one days the animals were killed and the circulation perfused with a mixture of barium sulphate and gelatin; radiograms were taken of the vascular trees of the two limbs. Whereas the main anastomotic channels were the same on the two sides, the network of fine vessels was markedly richer on the right, the side of venous ligature.

The immediate results of arterial cum venous and arterial ligature were compared by the study of the circulation in the frog's web. Ligation of the sciatic artery and femoral vein produced a far larger vascular bed than did ligation of the artery alone; the patent capillaries were more numerous, wider and loaded with corpuscles. Peripheral stagnation was more easily produced by simple arterial occlusion.

The further stages were studied in the unpigmented pads of kittens; on the side of arterial cum venous ligature, the pads were clearly redder than on the other side, whereas no temperature difference could be detected, *i.e.* the capillary bed was larger without any evidence of peripheral stagnation. Eight days later the kittens were killed and the vessels perfused with Prussian blue and glycerin; the muscles of the thigh and calf were removed and transverse sections were cut. Comparative counts of all the blood vessels in corresponding fields showed no definite numerical difference though the vessels on the side of the venous ligature were more prominent.

These experiments suggest that simultaneous ligature of artery and vein gives rise to a richer collateral anastomosis than in the case of simple arterial occlusion. This richness depends upon an increase in size of individual vessels rather than upon their number. The effect of venous obstruction is to maintain a larger vascular bed by diminishing the ease of escape of blood from the limb.

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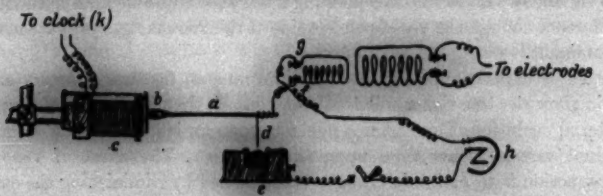
An Automatic Contact Breaker.

By Arthur Dighton Stammers.
University of the Witwatersrand, Johannesburg.

In studying the effect of fatigue in amphibian muscle and the influence of ultra-violet light thereon, it became necessary to employ a slow-moving drum, thereby rendering the ordinary automatic drum contact useless. In order to avoid the tedium of the observer sitting with a stop-watch and applying a stimulus at given intervals, the apparatus described below was devised. It can easily be fitted up in a few minutes from the resources of any physiological laboratory.

The apparatus consists of the ordinary requirements for obtaining a simple muscle twitch, plus some form of electric time-marking clock, a magnetic signal, a mercury contact and a short length of thin platinum wire.

A light extension (*a*) (see accompanying figure), about 3 ins. long, is fixed to the writing lever (*b*) of the magnetic signal (*c*). Round this



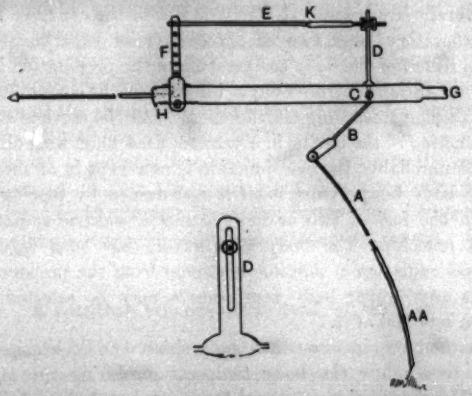
extension is wound two or three times a spiral of thin platinum wire. (Platinum is used as being more resistant to the sparking produced at the contact.) About an inch of free end (*d*) is left depending vertically. This is contrived so as to dip, when the current flows through the magnet of the signal, into the mercury contact (*e*), which is arranged immediately beneath it. The other end of the platinum wire is connected to one terminal (*f*) of the primary circuit of an induction coil. The other terminal (*g*) of the coil is connected to one pole of a battery (*h*) and the mercury contact to the other pole of the battery. Thus a key, comprising the magnetic signal with attached wire and mercury contact, is inserted in

the primary circuit of the coil. If the time-marking clock (*k*) is now connected to the magnetic signal, the movement of the latter will cause the circuit in the primary coil to be made and broken almost simultaneously by the momentary dipping of the wire into the mercury and the periodicity may be varied according to the capacity of the clock, *e.g.* 1 sec., 5 secs., 30 secs., etc.

The "make" effect of the current can be eliminated in the usual way, by adjustment of the secondary coil. It will then be found that the apparatus can be set up to give periodical "break" shocks for a considerable length of time without attention.

A Modification of Sherrington's Stethograph. By R. CAUNTER.
University of the Witwatersrand, Johannesburg.

The following details of a modification of the stethograph described by Sherrington[1], which has given very satisfactory results in use in this laboratory, are presented, since the present instrument embodies certain improvements which make for a more robust and, at the same time, a more easily adjustable unit.



The apparatus consists of a stiff wire stem, *A*, of which one end slides in a tube, *AA*, and the other is hinged to a lever, *B*. This in turn is fixed to a bearing, *C*, which is carried by a rod, *G*, which can be clamped to a standard. The bearing carries with it the lever, *D*, to which is attached

[1] *Mammalian Physiology*, Clarendon Press, 1919, p. 82.

an aluminium rod, *E*. The latter is connected, in the manner shown in the accompanying diagram, to a myograph lever, *F*, which is mounted on the other end of *C* and provided with a rack and pinion, *H*, to allow for fine adjustment of the writing point in the direction of the long axis of *C*. The lever, *D*, is slotted to allow for movement of *E*, thus providing for regulation of the amplitude of movement of the writing lever. Free movement of *E* is ensured by the swivel joint at *K*.

The advantages claimed are: (i) the apparatus is combined into a single unit; (ii) the wire stem can be lengthened to suit any height of drum by pulling out the metal tube which it carries for half its length; (iii) there is no tendency for slack, as when a thread is used to connect the stem portion to the myograph; (iv) the extent of movement of the writing lever may be easily regulated and (v) fine adjustment of the writing lever is provided.

A method for the automatic regulation of the body temperature of decerebrate and spinal preparations of the cat and other animals. By T. Graham Brown (*Cardiff*).

For several years decerebrate preparations of the cat have been used in this laboratory over two or three days of experimentation. A continuous record of the body temperature of the preparation is kept by means of an Adrian Thermo-Couple inserted in the abdominal cavity, and a Cambridge Recording Galvanometer. As the method of keeping the cold junction of the circuit in a vacuum flask filled with oil has been found to be unreliable, the cold junction is now kept in an incubator at 25° C. The body temperature itself is maintained by placing the preparation in the foci of two or more domestic electric radiators with paraboloid reflectors. The body temperature has been regulated by placing these radiators at suitable distances from the preparation. By this means any proper body temperature may be selected and the preparation retained at it.

This method requires constant supervision and it has been found impossible to regulate the body temperature during the night with certainty. The preparation may cool too much; or, as is more often the case, the temperature may rise too far and damage the preparation.

To overcome this difficulty the following method has recently been devised, and preliminary experiments show that it works satisfactorily:

A second Thermo-Couple is inserted in the preparation. The corresponding cold junction is placed alongside that of the recording thermo-

meter in an incubator kept at a constant temperature of 25° C. The electric circuit of the second Thermo-Couple passes through a "Cambridge Regulator" which in effect is a galvanometer combined with a delicate relay mechanism. This relay is set into action when the needle of the galvanometer comes exactly over a pointer which itself may be set at any point on the galvanometer scale. The relay circuit of the "Regulator" passes through another relay; and the circuit of this second relay passes through a third which makes and breaks the current through the electric radiators. If now the pointer on the "Regulator" be set on the scale at a chosen point (say that corresponding to 35° C.), the current passes through the radiators and the galvanometer needle moves up the scale with the rise in body temperature of the decerebrate cat. When the galvanometer needle comes immediately over the pointer the relay circuits are closed and the radiators are automatically cut off. The temperature of the preparation falls a little; and with this fall the galvanometer needle moves down the scale. The relay circuits are at once broken and the radiators are again set in action. The temperature of the decerebrate cat again rises—and so on.

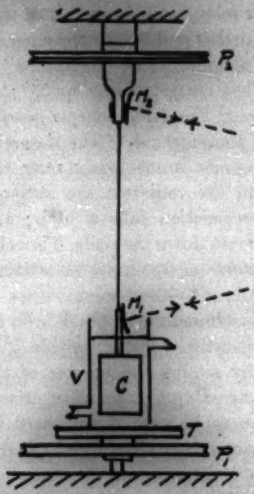
By means of this instrument it is possible to keep the temperature of the decerebrate preparation constant within $\frac{1}{2}$° C. on either side of a temperature selected by setting the pointer of the "Regulator." The apparatus has been so fitted up that an alternative Thermo-Couple kept at room temperature may be used if necessary for regulating by the temperature of the room rather than by the body temperature of the preparation. Variable resistances in the radiator circuits enable the radiators to set at suitable temperatures, and obviate the necessity of moving them to or from the preparation. The apparatus, which was supplied by the Cambridge Scientific Instrument Company, works efficiently and gives very constant and satisfactory results.

A rotation viscometer. By J. H. Shaxby.

A brass cylinder C is suspended with its axis vertical by a fine wire and hangs immersed in the liquid whose viscosity is to be investigated. The vessel V containing the liquid stands on a table T which is slowly rotated by a pulley P_1. The twist set up in the wire by the viscous forces is indicated by a mirror M_1 attached to the cylinder, and a lamp and scale.

If rotation of the vessel is inconvenient, *e.g.*, if it is fitted with inflow and outflow tubes, the cylinder is rotated instead of the liquid. The

suspending wire is attached at its upper end to the axle of a pulley P_2 which is slowly rotated, and a mirror fixed to a sleeve on the axle of the pulley is adjusted so that at each rotation the light spot it reflects upon the scale coincides with that from the mirror below. The distance upon the scale between the two light spots when the apparatus is finally brought to rest measures the twist of the wire.



A simplified model of Haldane's apparatus for the determination of O_2 and CO_2 in air. By M. E. MARSCHAK.

Laboratory of Labour Physiology, Moscow.

The Haldane apparatus for the determination of O_2 and CO_2 in air is one of the most widespread, and it enables thoroughly reliable and accurate determinations to be made within several minutes.

In addition to cleanliness, an absolute impermeability to gases is the principal condition for exact work with an apparatus for gas analysis; consequently as few taps and rubber connecting pieces as possible should be employed. We have attempted to satisfy this condition, while taking the utmost care that any changes introduced should in no way influence the accuracy of working[1].

[1] The first apparatus here described was prepared by the glassblower of the Institute, P. F. Feodorov.

The original Haldane apparatus for analysis of O_2 and CO_2 in air is provided with four taps: one tap—the main one—connects the graduated burette with the outer air and with the capillary tube from which branch off the absorption vessels for O_2 and CO_2; taps 2 and 3 are located on this capillary tube and connect the absorption vessels with the burette and outer air. Tap 4 is on the thermobarometric tube.

In our simplified model we have omitted taps 2 and 3 (Fig. 1). In order to attain this we modified the central tap on the calibrated burette.

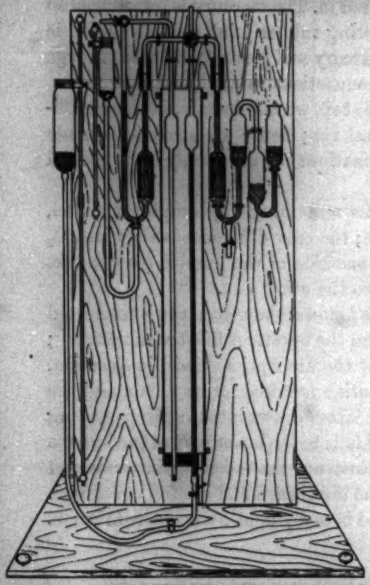


Fig. 1

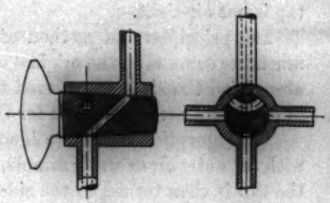


Fig. 2

The Haldane apparatus is provided with a central two-way tap; one way is used for taking in the air sample, the other connects the burette with the capillary tube united with the absorption vessels. The central tap in the modified apparatus is a three-way one: two side ways are joined with the absorption vessels and the third—sloping—is used to suck in the air sample (Fig. 2).

The absorption vessel for CO_2 is located, together with the thermobarometric tube, on one side of the graduated burette; the one for O_2

on the other side. Such a distribution and the absence of two taps render the apparatus more compact, and allow the possibility of constructing it entirely of glass. To prevent breakage, however, we have connected the central tap of the burette with the absorbing vessels by means of rubber joints with mercury seals (Fig. 1): a short glass tube (15—25 mm. internal diameter) is adjusted on the lower portion of the capillary tube leading to the absorption vessel, the lower end of this glass tube is firmly fitted with a rubber stopper and the upper end remains open to allow the mercury to be poured in. The mercury is introduced so as to cover both ends of the connecting rubber joint and a little oil is poured on top of the mercury. This mercury seal prevents diffusion through the rubber, or suction through the connections as often occurs.

Thus apart from the thermometric tap, we manipulate, in this simplified apparatus, only with the central tap; hence air analysis is made easier and suction during the determinations can occur only if the central tap is not air-tight.

The principles and method of use remain, in the simplified model, much the same as in the original one; the only difference is that for CO_2 absorption the air is transferred to one side of the burette (to the left, in the figure here given) and for O_2 to the other side.

The absorption vessels are filled as follows: the central tap is connected with the outer air or an air sample, and the burette is filled with mercury; the tap is then turned to the side of the absorption vessel in question, which is then connected with the solution required for its filling, and the mercury level is lowered. The solution is thus drawn into the corresponding side vessel. In case the mercury level falls to the lower part of the burette before enough solution has been sucked up, the tap is again connected with the air, the mercury is raised, the tap turned and suction continued. The procedure is not complicated and is easily done by one person alone.

A fixed-focus projector for the measurement of spectra.

By F. H. Gage.

The enlarger consists of a condensing lens and enlarging lens on the principle of the ordinary photographic apparatus.

The slide is placed on a carriage which can be moved along slowly by a worm reduction gear. A series of spectra can thus be projected consecutively for separate examination.

A small electric bulb lights up when the carriage reaches the end of its journey in either direction.

The magnification is fixed at exactly 1 inch to 1 cm. This is convenient for the construction of the wave-length scale.

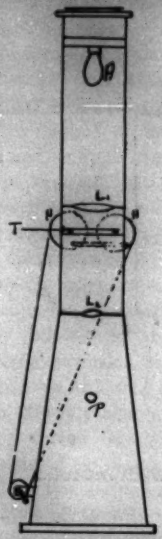


Fig. 1. Front elevation.

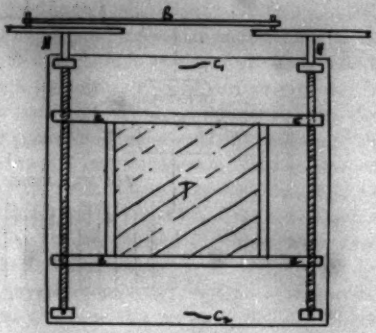


Fig. 2. Plan of carriage.

L_1, condensing lens; L_2, enlarging lens; T, spectrum plate; A, 100 watt lamp; P, small flash lamp; C_1, C_2, contacts which complete circuit when carriage *EEEE* reaches end of journey in either direction and causes P to light; H, H, pulleys which rotate screwed rods and thus move carriage slowly in the desired direction; B, "connecting rod" to keep pulleys H, H in step.

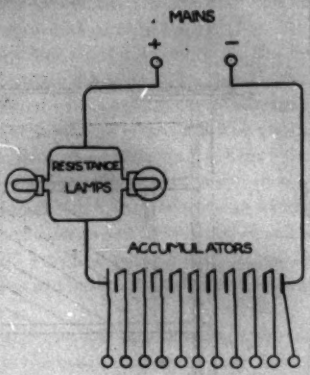
A method for supplying low-tension current.

By A. Hemingway (*Cardiff*).

If direct current mains are available a constant supply of low-tension current may be obtained by "floating" a series of accumulators across the mains, the current being regulated by a resistance in the live mains lead. Two volt tappings may then be made from individual cells of the battery.

The battery can be conveniently built into a box, the tappings being led to a terminal board. Lamps should be inserted into holders wired

in parallel and placed in the mains lead, the lamps being selected so that the inflow from the mains is approximately balanced against the current taken by the external circuit.



The combination of carbon dioxide with muscle.

By G. Stella (*Padua*).

In my paper on the combination of carbon dioxide with muscle(1) in referring to some experiments by Meyerhof(2) on the heat of combination of valerianic acid in muscle, I pointed out that the final concentration of this acid inside the muscles had not been determined, thus rendering the calculation of the heat of neutralization of the acid per mol. uncertain. I was unfortunately not aware of the fact that in later experiments by the same Author(3) this omission had been rectified, the heat having been estimated after a determination of the distribution of the valerianic acid between the Ringer's solution and the muscles.

The average value found in these experiments, *i.e.* 9900 calories per gm. molecule of valerianic acid neutralized, is very near to the value that I have given in my paper for the neutralization of carbonic acid in muscle, *i.e.* 9400 calories per gm. molecule.

1. Stella. This Journ. 68. p. 49. 1929.
2. Meyerhof. Pflüger's Arch. 195. p. 22. 1922.
3. Meyerhof. Ibid. 204. p. 295. 1924.

A class experiment in reciprocal contraction of antagonistic muscles. By GRACE BRISCOE.

(From the Physiological Laboratory of the London School of Medicine for Women.)

Reciprocal contraction of antagonistic muscles can be demonstrated very simply with ordinary students' apparatus, and the experiment is now part of the class routine in this laboratory.

The method of obtaining graded muscular contractions of natural form and its application to antagonistic muscles have been described in two papers (1) (2). Although the interruption of current provided by the ordinary induction coil is too rough to give satisfactory contractions for slow movements, yet the principle of reciprocal contraction and the fact that muscle can be caused to give movements other than sudden twitches are readily appreciated by the method here described. To obtain a record is a more troublesome matter, and as the movements of the limb can be easily followed by the eye, it has seemed better to concentrate the students' attention on the actual muscles.

The leg of a frog is skinned completely, but no other dissection is done except the isolation of the sciatic nerve as high up as possible. Damp threads are placed round the external and internal popliteal divisions of the sciatic, and these are gently pulled apart by the threads until the two nerves are separate at the upper part of the thigh. They are then cut off from the sciatic trunk, and protected from exposure. The limb need not be fixed. The usual induction coil apparatus for faradic stimulation is set up, but two complete secondary circuits with keys are provided. The secondary coils are placed on either side, at right-angles to the primary coil. One nerve is placed on a pair of electrodes, and the secondary coil concerned is placed close enough to the primary to give a small contraction. This procedure is repeated with the other nerve. The primary coil is then picked up and moved quietly to and fro between the two secondary coils.

As a result of this movement the induction currents supplied to the stimulating electrodes will vary continually in strength, but they will vary in an approximately reciprocal relation, so that when the flexor group of muscles is contracting strongly, the extensor group is almost completely relaxed and *vice versa*. In consequence the foot is moved alternately from a position of extreme flexion to one of extension and back again, as long as the to and fro movements of the primary are kept up. It is necessary to see that these to and fro movements are kept within the limits of just minimal and just maximal stimulation.

Alternatively the primary coil can be kept stationary, and the two secondary coils can be held one in either hand and made to approach and recede from the primary in a reciprocal fashion. In this method it is well to define and mark on th bench the useful limits of movement of each coil.

Again the secondary coils can be held in the air above the primary and be made to approach and recede reciprocally. This serves as a useful reminder that the varying field of magnetic force is not confined to one plane.

1. G. Briscoe. Quart. Journ. of Expt. Physiol. 19. p. 1. 1928.
2. G. Briscoe and W. A. Leyshon. Proc. Roy. Soc. B, 105. 1929.